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RETROVIRAL VECTORS FOR ANTI-HIV GENE THERAPY

by

DEBORAH RUTH (ROBINSON) HEMMERLING

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of the requirements for the degree of
DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY

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ABSTRACT

This thesis describes the development of a retroviral vector and anti-HIV genetic elements with potential therapeutic utility. A new retroviral vector was constructed based on Moloney murine leukemia virus (MLV). Alterations to the MLV provirus included replacement of a portion of the U3 LTR region with a hybrid regulatory element consisting of the human cytomegalovirus immediate early enhancer/promoter (CMV-IE) and the HIV transactivation response element (HIV-TAR). The hybrid LTR was cloned upstream of the chloramphenicol acetyl transferase (CAT) reporter gene. Transfection of this construct into a variety of human cell lines gave CAT levels which were 10- to 50-fold higher than from the wild-type MLV-LTR promoter. In addition expression from the hybrid LTR was increased in the presence of HIV-Tat protein. A modified retroviral vector containing the hybrid LTR plus an extended MLV packaging signal was transfected into the amphotropic packaging cell line PA317, it consistently gave higher titres of retrovirus than did the parental MLV vector. This novel retroviral vector should have advantages for anti-HIV gene therapy.

Packaging decoy elements (PAK140 and LJC140) were inserted into the improved retroviral vector (pTAR). Infectious recombinant viruses generated from these plasmids were stably transduced into two HIV susceptible human lymphoid cell lines. The resulting transduced cell lines were challenged with HIV-1 or 2 and cultures followed for virus production over a 40-day period. The cells transduced with the TAR-PAK140 virus were found to produce much less HIV-1 or HIV-2 than control transduced or untransduced cells. Cell lines which had been infected with HIV-1 and survived infection to become continuous producers of virus (i.e. 'chronically infected') were stably

transduced with TAR-PAK140 or control recombinant viruses. The resulting cell lines were monitored for production of infectious HIV-1 particles by titering 12-hour culture supernatants, and the supernatants were assayed for RT activity. The chronically infected cell lines transduced with TAR-PAK140 were seen to produce 10-fold fewer infectious particles over the assay period, but the RT activity was not correspondingly decreased. This suggests that more defective particles (perhaps co-packaged HIV and MLV RNAs) were being produced from the chronically HIV infected, TAR-PAK140 transduced cells.

Table of Contents

Chapter 1 - Introduction 1
A. Human Immunodeficiency virus and gene therapy 1
1. Overview 1
2. Retroviruses in general 2
3. General rules for retroviral infection 8
4. The specific case of HIV 9
a. Properties of HIV 9
b. Particle and genomic structure of HIV 9
c. Genomic structure 10
d. Essential Viral proteins 10
i. Gag, Pol, Env 10
ii. Tat 13
iii. Rev 15
e. The accessory proteins 15
i. Vif 16
ii. Nef 16
iii. Vpu 17
iv. Vpr 17
5. The specifics of HIV infection 18
a. Cell Tropism and Receptor Interaction 18
b. Attachment 18
c. Entry and uncoating 19
d. Replication 20
e. Latency and transcription activation 20
f. Encapsidation and assembly 22
6. HIV pathogenesis 23
7. AIDS 26
8. Anti-viral agents 27
9. Vaccines 29
B. Gene Therapy 30
1. Retroviral Vectors and Packaging cell lines 31
2. Specific problems of gene therapy for HIV 33
3. Limitations of MLV Based Vectors 34
4. Potential strategies for gene therapy of HIV infection 35
a. Protein based therapies-Trans-dominant anti-HIV proteins 35
b. Other protein based strategies 36
c. Drawbacks of protein based therapies 37
d. Non protein based therapy 37
e. RNA decoys 39
f. HIV packaging signal as a decoy RNA 40
5. Goals of this study 42

Chapter 2 - Modified Retroviral Vector 44
A. Introduction 44
B. Methods 45
1. Plasmid construction 45
2. Cell lines and viral strains 46
3. Transfection with CaPO4 (adherent cells) 46
4. Electroporation 48
5. Production of retroviral vectors 48
6. Generation of producer cell lines 49
7. Infection with virus produced from packaging cells 49
8. Titering virus produced from transfected packaging cell lines 49
9. PEG fusion 50
10. CAT assay 51
11. Northern analysis and RNase mapping 52
C. Results 53
1. Activity of MLV promoter with other viral enhancer/promoters 53
2. Reconstruction of the MLV LTR and testing of the hybrid promoter constructs 55
3. Levels of transcription from the two initiation sites of pMCT 64
4. Generation of retroviral vectors with modified LTRs 70
5. Relative virus titer of modified retroviral vectors 70
6. Levels of expression after long-term integration 71
7. Transactivation of the stably integrated MLV/CMV/HIV TAR LTR by PEG fusion with Tat-producing cells 75
D. Discussion 78
1. Gene therapy vector LTR modifications 78
2. Generation of high titer recombinant vectors 86
3. Overview of retroviral vector design changes 91

Chapter 3 - Anti-HIV elements 93
A. Introduction 93
B. Methods 94
1. Construction of HIV antisense expressing plasmids 94
2. Construction of the LJC140 and PAK140 retroviral vectors 97
a. LJC140. 97
b. PAK140 101
3. Oligonucleotide purification 101
4. Cell and virus strains 110
5. Kinetic assay for anti-viral effect 110
6. Cell viability staining 111
7. Reverse Transcriptase Assay 111
8. Surface analysis of cells expressing CD4 112
9. Immunostaining of HIV-1 and HIV-2 infected cells 112
10. Retroviral transduction 113

11. Northern blot 113
C. Results 114
1. Initial antisense constructs did not inhibit HIV virus production in a HeLa cell co-transfection assay 114
2. Two different HIV packaging decoy constructs were created: LJC140 (all sense strand) and PAK140 (mixture of sense and antisense). .. 118
3. A human lymphoblastoid cell line stably transduced with TAR-PAK140 gave rise to reduced levels of virus following HIV-1 infection. 122
4. TAR-PAK140 transduced H9 cells retained expression of CD4, whereas TAR-PAK140 transduced AA-2 cells showed reduced or even absent CD4 expression. 137
5. Higher levels of MLV vector-derived transcripts were found in the uninfected TAR-PAK140 transduced cell lines compared to the LSN-PAK140 transduced cell lines. 137
6. When cell lines chronically infected with tat-positive HIV-1_{NL4-3} were secondarily transduced with TAR-PAK140, production of infectious virus was reduced; this effect was not seen in the equivalent cell line infected with a tat-minus NL4-3 variant. 146
7. Chronically infected MT4.tat+$_{NL4-3}$ cells transduced with the LSN-PAK140 or TAR-PAK140 both showed high levels of MLV vector-derived transcripts, but these transcripts were nearly absent in the MT4.tat-$_{NL4-3}$ cells. 148
8. H9 cells stably transduced with TAR-LJC140 did not give rise to reduced levels of virus following HIV-1 or HIV-2 infection. 151
9. Kinetics of HIV-1 virus production were similar in parental H9 cells and H9 cells that had been stably transduced with proviruses containing LJC140+gag antisense elements or PAK140+ a gag antisense element. 151
D. Discussion 162

Chapter 4-Final discussion and conclusions 176

Appendix A-Construction of Thy containing vectors 234
A. Vector modification designed to increase long term stability of expression 234
B. Methods 235
1. Cloning of pLSNthycat and pLGCSTNthycat 235
C. Results 236
D. Discussion 242

Appendix B 243

List of Tables

Table 1. Cell lines 47

Table 2. Relative levels of promoter strengths of chimeric MoMLV LTR constructs *versus* HIV LTR and CMV promoter in different human cell lines. 61

Table 3. Titration of packaged retrovirus by G418 selection 72

Table 4. Oligonucleotides for PCR amplification of HIV-1 antisense constructs. 96

Table 5. Oligonucleotides used in the construction of the PAK100, LJC140 and PAK140 elements. 100

Table 6. Infectious virus produced over 12 hours from cell lines chronically infected with HIV-1 and then secondarily transduced with LSN-PAK140, TAR-PAK140 or TAR-CAT. 147

Table 7. Ratio of RT activity per infectious unit 173

List of Figures

Figure 1. Structural representation of a mature retroviral particle. 3

Figure 2. Reverse transcription of HIV RNA. 6

Figure 3. Diagram of the HIV-1 genome. 11

Figure 4. Comparison of heterologous promoter activities with the Moloney MuLV LTR. 56

Figure 5. Modification of the retroviral vector. 58

Figure 6. Effect of Tat (transient) on B lymphoblastomas expression from the pMT and pMCT constructs 62

Figure 7. pMCT-cat activity in CEM-TART cells which constitutively express Tat . . . 65

Figure 8. Analysis of transcription initiation from the pMCT-cat construct in HepG2 cells, in the presence or absence of Tat 68

Figure 9. Long-term study of expression from various recombinant vectors 73

Figure 10. Transactivation of integrated reporter gene by fusion to HeLa Tat cells 76

Figure 11. Details of the promoter elements of MLV LTR, pMT LTR and pMCT LTR 81

Figure 12. Diagram of the packaging signal in MFG, pLCSTN and pLGCTSN. 88

Figure 13. (**a**) Schematic representation of the 5' end of the viral genome, shown in expanded view. 98

Figure 14. Strategy used to clone pLLL-PAK100. 102

Figure 15. Strategy used to clone pLLL**gpt**-PAK100 and pLLL**gpt**-revPAK100. 103

Figure 16. Strategy used to clone pLLL**gpt**-LJC140.. 104

Figure 17. Strategy used to clone pLSN-LJC140.. 105

Figure 18. Strategy used to clone pTAR-LJC140. 106

Figure 19. Strategy used to clone pLLL**gpt**-PAK140. 107

Figure 20. Strategy used to clone pLSN-PAK140. 108

Figure 21. Strategy used to clone pTAR-PAK140. 109

Figure 22. Virus production from HeLa cells transfected with an HIV-1 proviral plasmid (pNL4-3) together with various antisense constructs 115

Figure 23. Comparison of pTAR-LJC and pTAR-PAK140. 119

Figure 24. (**a**) DNA sequences of the synthetic HIV-1 packaging elements PAK100 and LJC140. 120

Figure 25. Kinetics of HIV virus production in wild-type and transduced H9 cells infected with HIV-1_{NL4-3}. 123

Figure 26. Kinetics of HIV virus production in wild-type and transduced H9 cells infected with HIV-1_{ELI}. 129

Figure 27. Kinetics of HIV virus production in wild-type and transduced AA-2 cells infected with HIV-1_{NL4-3}. 131

Figure 28. Kinetics of HIV virus production in wild-type and transduced H9 cells infected with HIV-2_{ROD}. 135

Figure 29. Kinetics of HIV virus production in wild-type and transduced AA-2 cells infected with HIV-2_{CBL}. 138

Figure 30. FACScan analysis of wild-type and transduced H9 cells stained with anti-human CD4 antibody. 140

Figure 31. FACScan analysis of wild-type and transduced AA-2 cells stained with anti-human CD4 antibody. 142

Figure 32. Autoradiograph of a Northern blot 144

Figure 33. Northern analysis of retroviral RNAs 149

Figure 34. Kinetics of HIV virus production in wild-type and transduced H9 cells infected with HIV_{NL4-3}. 152

Figure 35. Kinetics of HIV virus production in wild-type and transduced H9 cells infected with $HIV\text{-}2_{CBL}$. 155

Figure 36. 158

Figure 37. Kinetics of HIV virus production in wild-type and transduced H9 cells infected with $HIV\text{-}1_{1648}$. 160

Figure 38. Construction strategy of pks+5'LGCTSN. 237

Figure 39. Construction strategy of pks+5'LGCTSN+Thy. 238

Figure 40. Construction strategy of pLGCSTN.Thy-cat. 239

Figure 41. 240

Abbreviations

AB	Alberta
AIDS	Acquired Immunodeficiency Syndrome
ADCC	Antibody dependent cell-mediated cytotoxicity
AP-1	Adapter protein
AZT	3'-Azidothymidine
BBS	BES/NaCl/Na_2HPO_4-transfection buffer
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
CA	capsid
Can	Canada
CAT	Chloramphenicol acetyl transferase
CD	Cluster of differentiation
Cf	Final concentration
CMV	Cytomegalovirus
CMV-IE	CMV-immediate early
CNS	Central nervous system
CTL	Cytotoxic T-lymphocyte
ddC	2',3'-Dideoxycytidine
ddI	2',3'-Dideoxyinosine
DIS	Dimerization initiation site
dl.CMV-IE	Deleted.CMV-immediate early
CTD	Carboxyl terminal domain
DLS	Dimer linkage structure
dsDNA	Double stranded DNA
DMEM	Dulbecco's minimal eagle medium
EBP-1	enhancer binding protein

EDTA	Ethylene diamine tetra-acetic acid
env	Envelope
EBV	Epstein Barr virus
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
g	Gravities
gag	Group associated antigen
h	Hour
HIV	Human immunodeficiency virus
IL	Interleukin
IN	Integrase
IU	Infectious units
JC	Jamestown Canyon
LTR	Long terminal repeat
MA	Matrix associated
µl	Micro liter
min	Minute
mg	Milligrams
ml	Milliliter
MLV	Moloney murine leukemia virus
MLV-A	MLV-amphotrohic
MLV-E	MLV-ecotrophic
MLV-X	MLV-xenotrophic
mM	milli-Molar
MOI	Multiplicity of infection
MOPS	3-morpholino-propanesulfonic acid
mRNA	messenger RNA

MSV	murine sarcoma virus
M-tropic	Macrophage-trophic
NC	Nucleocapsid
NEB	New England biolabs
Nef	Negative factor
NF-κB	Nuclear factor κB
NK	Natural Killer
NIAID	National Institute of Allergy and Infectious Disease
NIH	National Institute of Health
NSI	Non-syncitia inducing
Ont	Ontario
PBMC	Peripheral blood monocytic cell
PBS	Phosphate buffered saline
pbs	Primer binding site
PCR	Polymerase chain reaction
PEG	Poly-ethylene-glycol
pol	Polymerase
psi ψ	Packaging signal
PR	Protease
Que	Quebec
R	Repeat
Rev	Regulator of viral expression
RSV	Rous sarcoma virus
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RRE	Rev response element
RT	Reverse Transcriptase

sCD4	Soluble CD4
SDS	Sodium dodecyl sulfate
SFv	Single-chain variable fragments
SI	Syncitia inducing
SIV	Simian immunodeficiency virus
SU	Surface
TAR	Transactivation response element
Tat	Transactivating
TCID	Tissue culture infective dose
TE	Tris/EDTA
TEN	Tris/EDTA/Sodium chloride buffer
TFIID	Transcriptional factor II D
TGF	Transforming growth factor
TM	Transmembrane
TNF	Tumor necrosis factor
T-tropic	T cell trophic
tRNA	Transfer RNA
U3	Unique 3' region
U5	Unique 5' region
UBP-1	untranslated region binding protein
URE	Upregulatory element
URS	upstream repressing sequence
USF	upstream stimulating factor
VRC	vanadyl ribonucleoside complex
V3	Third variable region of gp 120
Vh	Variable heavy
Vif	Virion infectivity factor

Vl	Variable light
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VSV	Vesicular stomatitis virus

CHAPTER 1 - INTRODUCTION

A. Human Immunodeficiency virus and gene therapy

1. Overview

Human immunodeficiency virus (HIV) is a human pathogen that is the etiological agent of acquired immunodeficiency syndrome (AIDS) (20). Millions of people are infected with this retrovirus and most will die. The loss and potential loss of human life is only one of the significant effects of this virus; HIV has also affected general health care, and the development of new drugs. Interest in this pathogen has driven research into its biology in an unprecedented manner.

Although the scientific progress that has been made since HIV was first identified is remarkable, the nature of HIV and its infective cycle has posed some unique challenges for researchers trying to design cures and controls. One unusual property of HIV which makes it particularly difficult to eradicate is its ability to integrate into the chromosome of it's host cell. Therefore, although the amount of work done on HIV/AIDS is truly impressive there is still much that needs to be done. At this time there is no cure for AIDS and no vaccine against HIV.

Of the available anti-HIV drugs the newest, the protease inhibitors, have offered real hope in the war against HIV. The inherent problem with any anti-viral drug such as protease inhibitors is the need for a lifelong drug regimen. An innovative solution to this specific problem is the use of gene therapy wherein the introduction of a therapeutic gene could be of lifelong benefit to an AIDS sufferer.

Gene therapy is on the cutting edge of medical science. It involves the introduction of new genetic material into an organism for therapeutic benefit. Ideally the new genes would be introduced stably into the chromosome. It is ironic that retroviruses are convenient vehicles for the delivery of genetic material because of the property of genome insertion. Gene therapy using retroviral based vectors is currently being studied by clinical trial. As the cause and cure involve similar processes, the introduction of this thesis begins with a discussion of the general properties of retroviruses followed by a look at the specifics of HIV/AIDS and gene therapy vectors.

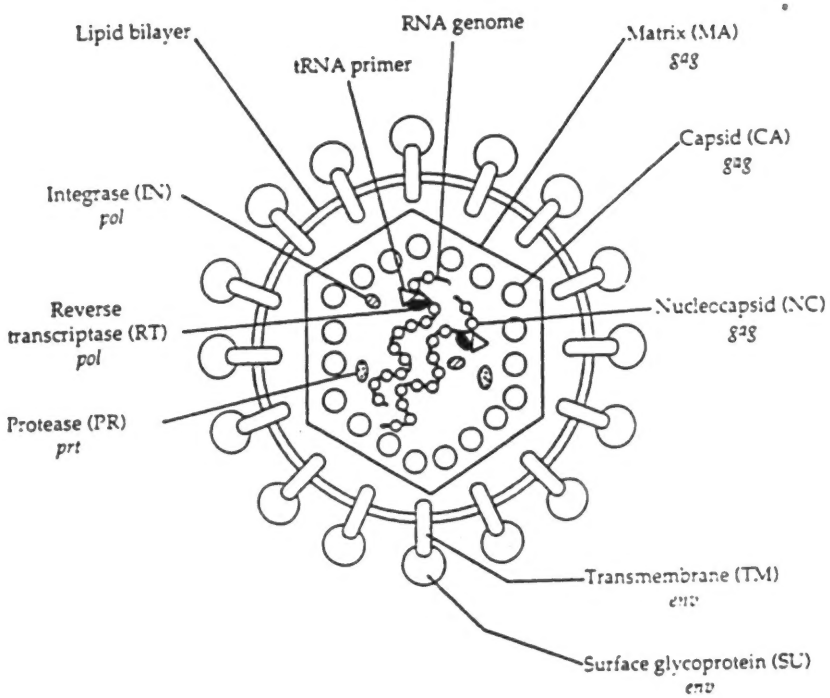
2. Retroviruses in general

Retroviruses have been extensively studied, not only because of HIV/AIDS but because members of the family cause tumors in animals. This tumor causing effect is the result of two mechanisms: 1) some retroviruses contain an oncogene and 2) the insertion of viral promoter elements next to a cellular proto-oncogene can lead to inappropriate expression of that gene (insertional mutagenesis)(363).

The family *Retroviridae* is divided into three subfamilies based on pathogenicity: *Spumavirinae* known as the foamy viruses, *Lentivirinae* for slow viruses and the *Oncovirinae* or tumor viruses (79). HIV belongs to the *Lentivirinae* while the viruses generally used for gene therapy are *Oncovirinae.*

Retroviruses are spherical particles, 80-100 nm in diameter with a unique three layered structure (Figure 1) (79). They are enveloped RNA viruses, with the RNA contained in a proteinaceous core (79). The envelope is derived from the plasma membrane of the virus-producing cell and is covered with glycoproteins (79). The core consists of an

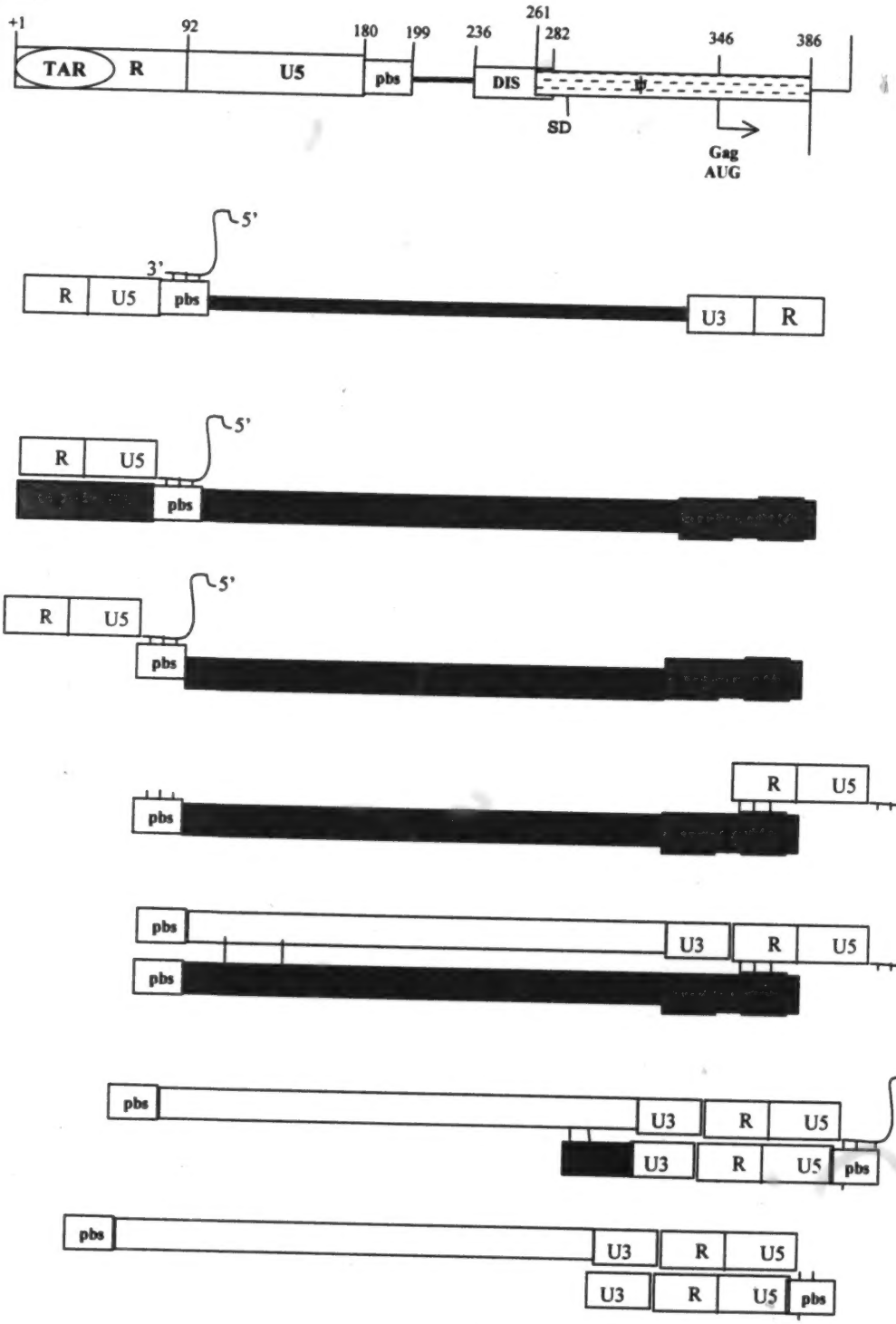
FIGURE 1. Structural representation of a mature retroviral particle.


Lipid bilayer
RNA genome
tRNA primer
Matrix (MA)
gag
Capsid (CA)
gag
Integrase (IN)
pol
Reverse
transcriptase (RT)
pol
Nucleocapsid (NC)
gag
Protease (PR)
prt
Transmembrane (TM)
env
Surface glycoprotein (SU)
env

icosahedral capsid and is separated from the envelope by matrix proteins (79). Contained within the capsid is the genome complexed with viral nucleoprotein. The nucleoprotein species include the protease, reverse transcriptase and integrase enzymes. The retroviral genome is unique among viral genomes: 1) as it is the only known diploid viral genome 2) the viral RNA is synthesized and processed completely by cellular machinery 3) the genome associates with a cellular tRNA and 4) it has the only positive-stranded genome that is not used as an mRNA template immediately after infection. The significance of the latter point is not known. Each haploid segment is a linear, single stranded, positive sense RNA with a 5' cap and 3' poly A (79).

The primer binding site (pbs) of the RNA is base paired to a particular cellular tRNA. The pbs in the viral genome is complementary to the 3'-terminal 18 nucleotides of the primer tRNA molecule (203). Reverse transcriptase initiates DNA synthesis by extension of the 3'-OH of the tRNA molecule that is bound in this position (340). The retroviral genome is reverse transcribed into linear double stranded DNA which contains long terminal repeats (LTRs). LTR's are derived from unique sequences at each end of genomic RNA (203). The HIV LTR is defined by three regions; U3, R and U5. The names of these elements are derived from their location in the viral genomic RNA. The genome contains a unique 3' region (U3) followed by the R or repeat section, the 5' end contains the R region followed by sequences unique to the 5' end (U5). After the RNA is reverse transcribed into the DNA provirus the U3 and U5 regions become duplicated to create the LTR (Figure 2). Viral DNA is integrated into host-cell DNA and the integrant consists of short inverted repeats that are bounded by the dinucleotides 5'TG.....CA 3'. Direct repeats of 4-6 bases flank the provirus,

FIGURE 2. Reverse transcription of HIV RNA. Reverse transcriptase elongates from the 3' end of the tRNA molecule to the 5' end of the viral RNA to produce strong-stop DNA that has minus strand polarity. All minus strand DNA species are represented by a broken line. 2) RNase H (RN) a domain of the RT protein degrades part or all of R from the 5' end of the viral genome thus creating a region of single stranded DNA referred to as R'. 3) The exposed R' hybridizes with the complementary R sequence at the 3' end of the second RNA molecule. 4) RT elongates from the strong-stop DNA and copies the viral genome. 5) RNase degrades viral RNA in the hybrid at the border with the 3' U3 sequence and generates an oligonucleotide primer designated as the plus strand primer (PB+). PB+ is a stretch of 12-15 purines known as the polypurine tract (PPT). From PB+, RT copies minus-strand strong-stop DNA. All plus-strand DNA species are represented by a shaded line. 6) The tRNA primer in the hybrid is removed by RNase H and in this way the PB- sequence is exposed. The second strand transfer is an intramolecular event in which the PB- in the plus strand DNA forms a duplex with the complementary sequence at the other end of the minus strand DNA molecule. 7) RT elongates from PB- in both directions. A strand displacement event separates the duplex between U3, R, U5 and U3', R', U5'. Accordingly, the long terminal repeats (LTRs) are generated as RT continues to elongate through these displaced sequences until the ends of the templates are reached. The product of reverse transcription is a fully duplex, linear DNA molecule with LTRs and blunt termini. This form of viral DNA is in a cytoplasmic nucleoprotein complex that contains integrase (IN) and other virion proteins.


+1
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261
282
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DIS
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pbs

and the size of the repeat depends on the virus (203). The genome of all nondefective retroviruses contains three major genes, each coding for two or more polypeptides (79). The *gag* gene (group specific antigen) encodes the virion core proteins. The three mature *gag* gene products common to all retroviruses are designated matrix (MA), capsid (CA) and nucleocapsid (NC). The *pol* gene (polymerase) encodes the enzymatic proteins reverse transcriptase (RT), integrase (IN) and protease (PR) and the *env* gene (envelope) encodes a polyprotein that is post-translationally modified in the endoplasmic reticulum by cleavages and glycosylation events to yield a surface (SU) domain and a transmembrane (TM) domain (79). The greatest degree of viral strain variation is observed in the env genes of closely related retroviruses and independent isolates of the same virus (91).

3. General rules for retroviral infection

The viral envelope attaches to the host cell surface receptor via the SU glycoprotein. TM catalyses a membrane fusion event which releases the viral core into the cytoplasm of the target cell (79). Reverse transcription occurs early during infection in a nucleoprotein complex in the cell cytoplasm (Figure 2) (203). Using the tRNA bound to the primer binding site as a primer, reverse transcriptase converts the genomic RNA into dsDNA. The priming tRNA is complementarily bound to a sequence in the viral genome called the minus-strand primer binding site (-pbs)(203). The reaction occurs as the genome is being transported to the cell nucleus.

Random integration of the provirus follows nuclear transport. Integration is non-homologous with some degree of sequence specificity and a preference for transcriptionally

active sites (242, 316). As a result of integration, infected cells shed virus continuously. For most retroviruses, virus shedding does not harm the cell (79).

After integration of the double-stranded DNA provirus, regulatory promoter and enhancer elements in the U3 region of the proviral LTR drive transcription of the viral genome (203). Gag and gag-pol proteins are transcribed from the full length mRNA. Smaller spliced transcripts are responsible for the production of the env proteins (350). The capsid proteins self assemble by binding to the matrix protein which is attached to the viral membrane. Full-length unspliced viral mRNA binds through its packaging sequence to the gag polyprotein and the env proteins are incorporated into the viral progeny as they bud from the cell surface. Newly budded virions are not infectious until the viral protease cleaves the gag and gag-pol polyproteins into their component parts (326).

4. The specific case of HIV

a. Properties of HIV

Two types of HIV are currently recognized, HIV-1 and HIV-2. HIV-2 displays 40% nucleotide sequence homology with HIV-1 and contains an unique gene, vpx in lieu of vpu of HIV-1 (Figure 3). Nine HIV-1 genetic subtypes or clades (A to I) have been designated within the major (M) group. These differ by 30-35% in their env and gag sequences. In addition there is also a more divergent set of viruses known as the outlier (O) group (385).

b. Particle and genomic structure of HIV

The envelope of HIV has 72 peplomers. Peplomers are viral proteins that project from the outer surface of the virus. HIV's peplomers are derived from the cleavage of HIV gp160 *(env* gene product) into two non-covalently linked components gp120 and gp41 (155).

Gp120 contains the receptor-binding domain and important antigenic domains. Gp120 is the most extensively glycosylated viral protein known, 55% of its molecular mass is composed of N-linked carbohydrates (43). The N-linked carbohydrates may act as hindering structures that limit antibody recognition of the virus. The amino terminus of gp41 contains a fusion peptide structure (53, 191). gp41 anchors the viral peplomer to the viral membrane.

The inner surface of the envelope is lined by a myristylated matrix protein, p17. This is a cleavage product of the 55kDa gag gene product (135). The capsid is composed of the phosphoprotein p24. Two other viral proteins, p9 and p7, are associated with the genome at the core of the particle (135). Four enzymatic functions are associated with the core, reverse transcriptase and its associated RNAase H, integrase and protease (155).

c. Genomic structure

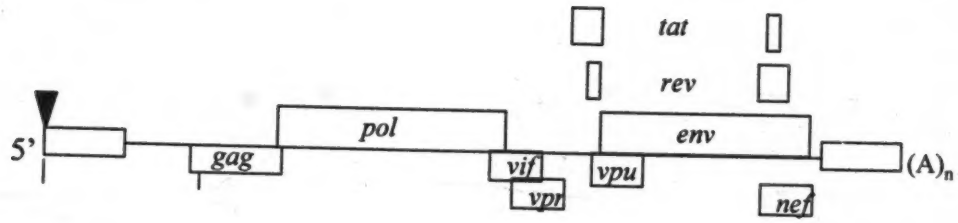
The genome of HIV (approximately 9.3kb) consists of two identical chains of RNA coupled to a tRNA (350). HIV and SIV(simian immunodeficiency virus) contain the three genes common to all retroviruses as well as six other genes not found in other retroviruses. The genome also includes the sequences required for packaging retroviral RNA and splice acceptor and donor sites for the generation of less than genome length RNA (Figure 3) (89).

d. Essential Viral proteins

i. Gag, Pol, Env

The *gag* gene encodes the virion proteins which form the inner matrix and core of the viral particle. *Gag* is synthesized as a 55,000-molecular-weight precursor, $Pr55^{gag}$ and forms the structural basis of the viral core. During or after budding $Pr55^{gag}$ is cleaved by

FIGURE 3. Diagram of the HIV-1 genome.


tat
rev
5'
pol
env
gag
vif
vpu
vpr
nef
(A)n

the viral protease to yield the viral proteins p17(matrix; MA), p24(capsid; CA), p7 (nucleocapsid; NC), and p6 (135).

Roughly 5 to 10 % of the ribosomes translating the *gag* gene undergo a frameshift near the p7/p6 junction, resulting in the deletion of the p6 domain and production of a much larger Gag-Pol fusion protein precursor ($Pr160^{gag\text{-}pol}$) (163, 266). The inclusion of the gag portion at the amino end of the polyprotein precursor allows the *pol* gene products to be incorporated into the assembling viral particle. Cleavage of the gag-pol precursor gives rise to three enzymes, protease, reverse transcriptase, and integrase, which are all necessary for viral replication (203).

The envelope glycoprotein is synthesized as a polyprotein (gp160) that is subsequently cleaved to produce the homodimer complex gp120 and gp41 (155).

In addition to the three prototypic retroviral proteins there are six other proteins produced by HIV-1; Tat, Vif, Vpr, Rev, Vpu and Nef. HIV-2 encodes all but Vpu and instead produces a protein called Vpx. Tat and Rev are absolutely essential for virus growth in vivo (89).

ii. Tat

Tat is a small nuclear protein of 86 to 101 amino acids, (size is strain dependent) that is required for the efficient synthesis of HIV proteins. The HIV-1 and HIV-2 Tat proteins contain a number of distinct domains, including amino-terminal, cysteine-rich, core, basic and auxiliary domains (54). The basic domain, amino acids 48 and 57, mediates Tat binding to TAR, and also functions in nuclear localization (51, 318). The conserved cysteine-rich region (amino acids 27 and 37) is important for protein-protein interactions (283).

It is well accepted that Tat is required for optimal HIV viability (95, 122) and it functions as a transcriptional activator from the HIV LTR (194, 272, 282). Tat both activates transcriptional initiation (194, 324) from the 5' LTR promoter and enhances the processivity of the elongating transcription complexes (117). Tat is different from conventional DNA binding transcription factors; most activators affect transcription by increasing the rate of initiation, in contrast Tat predominantly stimulates elongation (90, 126, 134, 169). As a transcriptional activator it is active on RNA and binds to the nascent leader mRNA (30, 51, 101, 296). Tat binds to an RNA stem-loop structure encoded by the transactivation response element (TAR) (101). TAR is located downstream from the transcriptional start site in the HIV-1 LTR, within a region of dyad symmetry which extends from +1 to +59. The specific binding of Tat to TAR is dependent on the bulge loop and immediate flanking sequences in the double-stranded RNA. Cellular proteins that recognize the loop sequence appear to be essential for Tat function on the natural TAR (377). Tat behaves as an anti-terminator by binding to the TAR RNA along with cellular co-factors to create a modified transcription complex (174, 178).

Tat has multiple roles that directly or indirectly affect AIDS pathogenesis. Tat can also transactivate heterologous viral (69, 181, 347) and cellular genes and alter the functions of host cells (50, 112, 302, 303, 384). For instance in acutely or chronically infected CD4 T cells Tat activates the expression of the tumor necrosis factors α and β (TNF α and β) (50). TNF is implicated in the pathogenesis of HIV in the central nervous system (165). TNF also activates HIV-1 gene expression (21). Tat is secreted by productively infected cells (127). Extracellular Tat can be taken up by chronically infected cells, transactivate transcription

from the HIV-1 LTR and induce reactivation of latent infections (112, 153). Extracellular Tat can inhibit the proliferation of antigen- stimulated T cells (334, 355) and can activate the production of transforming growth factor (TGF) ß in bone marrow-derived macrophages (384). TGF-ß is a potent suppresser of immune and myelopoietic systems (137) and its induction by Tat plays a role in the pathogenesis of the disorders affecting these systems. Tat has become an important target for anti-HIV therapy because of its primary role in the maintenance of HIV's life cycle and the other secondary effects attributed to it.

iii. Rev

Rev is a 116 amino acid phosphoprotein responsible for the temporal expression of HIV-1 structural proteins (182, 216, 217, 220, 221). Rev localizes to the nucleus of expressing cells (88) and binds to a region of complex RNA structure, the Rev-response element (RRE). RRE is present in the env regions of all singly and unspliced viral transcripts (94, 95, 146, 221, 345, 383). The Rev-RRE interaction activates the incompletely spliced transcripts for nuclear export through an active transport pathway (40, 120, 123, 333). Recent evidence indicates that Rev can shuttle between the cytoplasm and the nucleus (231). A nuclear pore protein may be a critical target for Rev interaction (40, 123). Rev is a good candidate for anti-viral therapy as its proper function is essential for a productive viral infection.

e. The accessory proteins

The accessory proteins vif, nef, vpu, and vpr are not absolutely required for the in vitro propagation of HIV. However they are important for optimal viral replication and essential for disease pathogenesis of the virus.

i. Vif

Virion infectivity factor (Vif) affects the formation of functional virus. Deletions in the *vif* gene have been associated with loss of viral infectivity in certain types of cells. Whether a cell is 'permissive or nonpermissive' for Vif-strains of HIV is determined by the cells that produce the virus (128, 357). Vif is essential for virus replication in peripheral blood mononuclear cells (PBMC), macrophages and some T lymphoid cell lines such as H9 (7, 113, 128, 357). In PBMC, virus produced by Vif- lines of HIV are 1000X less infectious than when Vif is present (121, 330). In HIV-1, Vif expression is Rev dependent and occurs late in the viral infective cycle (132, 306).

Although small amounts of Vif can be detected in viral particles (208) it remains to be demonstrated that its inclusion in the virus is important for infectivity (52). In fact Vif's role in regulating the infectivity of viral particles is unknown.

ii. Nef

The term Nef is an acronym for negative factor as this protein was originally thought to down regulate transcription from the HIV LTR (3, 254). This initial discovery was made using virus grown in culture and was not reproducible by all groups (147, 182). Nef has many subtle effects on viral replication and pathogenesis and has been shown to be a positive factor *in vivo* (179). Nef is critical for the establishment and maintenance of a pathogenic SIV infection *in vivo*.

Nef is predominantly a cytoplasmic protein that associates with the plasma membrane through an N-terminal myristate (147). Although its exact role in HIV replication is unknown at least three effects can be ascribed to Nef; The induction of CD4 downregulation

(5), the alteration of T cell activation pathways (213, 253, 320) and the enhancement of virus infectivity (320, 328).

iii. Vpu

Vpu (viral protein U) is expressed late in the HIV infective cycle. It is found in most HIV-1 isolates but not HIV-2 or SIV, except for SIV_{cpz}. Vpu is an integral membrane phosphoprotein that associates mainly with the internal membranes of the cell, apparently in an oligomeric form. Vpu down modulates CD4 (369), and enhances virus release (331, 332, 343).

iv. Vpr

Vpr (viral protein R) is found in HIV-1, HIV-2 and most SIV isolates. Vpr is a short basic protein that associates with viral particles through an interaction with the C-terminal region of gag and accumulates in the nucleus of infected cells (211). Vpr is functional in two processes: it is part of the machinery that allows HIV to infect non-dividing cells (110) and it prevents cells from passing into mitosis (22, 151, 171, 279, 291). Vpr produces an accumulation of cells in the G2 phase of the cell cycle and thereby prevents the establishment of chronic HIV-1 infection in T lymphocytes (291). It is not clear why the virus would induce this effect in infected cells.

Vpr is very important because it, along with the MA protein, enables HIV to infect non-dividing cells (152, 356). Vpr along with MA connects the preintegration complex with the cell nuclear import pathway.

5. The specifics of HIV infection

a. Cell Tropism and Receptor Interaction

Most retroviruses with the exception of the lentiviruses cannot infect non-dividing cells (204). Oncoretroviruses depend on cell proliferation for their replication, because the breakdown of the nuclear envelope at mitosis allows the viral preintegration complex to interact with the host cell chromosomes. HIV does not need to rely on nuclear membrane breakdown because it has proteins that guide the replicating provirus to the nucleus during infection (106).

HIV can infect activated T lymphocytes, monocytes, macrophages, dendritic cells, and microglia cells in the central nervous system (133, 186, 275, 364). All HIV-1 isolates replicate in primary T lymphocytes. Monocytes and transformed T cell lines are variably infected by primary HIV isolates. The tropism of the viral isolates from a patient usually relates to the stage of viral infection. In the early stages of infection, primary viral isolates replicate in monocytes and macrophage cells but not in transformed T-cell lines (M-tropic) (66). As HIV infection proceeds the M tropic viruses are usually replaced by T-cell-tropic types (T-tropic) (83, 293, 387). The T-tropic viruses infect primary and transformed T lymphocytes but not monocytes/macrophages. Dual tropic viruses are occasionally isolated (103). The transition of M tropic to T tropic viruses usually marks the onset of AIDS in the infected individual (66, 83, 305, 341, 387).

b. Attachment

For HIV to enter a cell it must sequentially engage two receptors on the cell surface, the first of which is the CD4 protein. Most isolates of HIV so far identified use this as a co-

receptor for entry into target cells (93, 185). The HIV envelope glycoprotein gp120 binds to the CD4 amino terminal domain (154, 227). After CD4 was identified as an entry determinant for HIV infection it soon became apparent that the CD4 molecule was not sufficient to allow infection (15, 214, 256).

The differences in tropism observed for the varieties of HIV can be accounted for by the use of a second receptor. The chemokine receptors CXCR4 (119) (formerly fusin) and CCR5 (11, 97, 105) are the main coreceptors for HIV-1 entry into CD4+ cells (T-tropism). Non-synctia inducing viruses (NSI), which are macrophage-tropic use CCR5, whereas syncitia inducing (SI) strains are adapted to T-cell lines and use CXCR4. In addition CCR1, CCR2b and CCR3 can function to a limited extent as co-receptors for some viral strains (103). This group of co-receptors are all chemokine receptors and share a common structure (ie: they are all seven-transmembrane (7TM) glycoproteins); they function to mediate the chemotaxis of T cells and phagocytic cells to areas of inflammation. The viral determinant of tropism has been shown to be conferred by the HIV-1 envelope (67, 68, 255, 317, 366). The third variable region or V3 loop of gp120 is responsible for receptor attachment.

CD4+ is the principle cellular receptor for type 1 HIV (HIV-1) but several CD4-negative cell lines are susceptible to infection with one or more HIV strains. In human glial and colon carcinoma cells HIV entry is mediated by galactosyl ceramide (148). HIV-2 can use CXCR4 as a primary receptor when infecting CD4-negative cells (111).

c. Entry and uncoating

The binding of the CD4 to gp120 leads to a conformational change in the viral protein allowing gp120 to bind to a second receptor (348, 376). After the gp120 binds to the second

receptor the viral membrane fuses with the host cell membrane. Fusion is mediated through gp41 and viral membrane fusion with host cell membrane follows (154, 191, 329), although how the virus initiates fusion with the host cell is not clearly understood. Viral entry can be one of two types, pH dependent or pH independent (367). pH dependent entry involves receptor mediated endocytosis and pH independent entry is through direct fusion of the virus with the cell membrane. It is known that the entry of HIV is pH independent (329) and therefore it seems likely that the gp41 glycoprotein is fusogenic in its native form.

d. Replication

After entry of the viral core into the cytoplasm, RNA is transcribed into double stranded DNA by the RNA/DNA-dependent DNA polymerase and ribonuclease H activities of reverse transcriptase (79) as described earlier. All HIV types initiate transcription from a $tRNA_3^{Lys}$ (164). The reverse transcriptase copies the first 18 nucleotides of the still-attached tRNA primer during plus-stranded strong-stop DNA synthesis (203). Reverse transcriptases are error prone; HIV's own reverse transcriptase has a high error rate with a frequency of misincorporation ranging from 1/1700 to 1/4000 (278, 289). After reverse transcription the integrase function of the core randomly integrates linear DNA into the cell chromosome (114).

e. Latency and transcription activation

The integrated provirus may remain latent indefinitely. Transcription of HIV cannot occur in resting cells but only in replicating cells (228, 382). Activation of the T cell can be brought about by mitogens (125, 381, 382), cytokines, or transactivating proteins encoded by other viruses. Many of these agents activate the cell by activating expression or

derepression of members of the NF-κB gene families (102, 249). This results in the transport of NF-κB to the nucleus where it interacts with enhancer elements to direct transcription from cellular genes. The HIV LTR contains two κB enhancer elements that are activated by the presence of NF-κB and this enables the viral genome to be transcribed at low levels (249). Transcription is a complex process involving interplay between cis-acting regulatory sequences and trans-acting cell and viral factors. The viral LTR has sequences that are typically used by RNA polymerase II. The promoter element contains a TATA box recognized by TFIID (130), three GC-rich sequences which bind to Sp1, (168) and an upregulatory element (URE) (252). In the untranslated leader region repeats are recognized by the cellular protein UBP-1 (130) and the trans activation response element (TAR). The enhancer is recognized by EBP-1, NF-κB (375) and HIVEN 86A. Sequences with the potential to decrease the synthesis of viral RNA include the negative regulatory element recognized by factors including AP-1, NFAT-1,URS and USF (130, 311).

Three major classes of HIV mRNA are produced, while multiple splicing options produce over 30 distinct RNA species (350). Unspliced RNA is the viral genome and is used as an mRNA for the translation of Gag and Pol polyproteins. Singly spliced 4.5 kb RNAs encode env, vpr and vpu proteins, while several 2 kb multiply spliced transcripts encode Tat, Rev and Nef proteins (350).

Transcriptional activation mediated through promoter sequences in the long terminal repeat gives rise to multiply spliced, small RNAs that code for the regulatory proteins Tat, Rev and Nef. As previously stated, the Tat protein is a transcriptional activator that interacts with the TAR structure present on the 5' end of all viral RNA transcripts. Tat greatly

enhances the efficiency of transcription from the HIV promoter. The Tat/nucleic acid interaction gives rise to large quantities of spliced viral RNA. When Rev has accumulated to a sufficient amount it interacts with the RRE (350). This interaction inhibits splicing and promotes transport of the singly spliced and unspliced RNA to the cytoplasm. RRE is located within the env gene and is present in the 9.2 and 4.5 kb HIV transcripts but not in the multiply spliced types, Rev also functions in the translation of these messages and promotes the transport of singly and unspliced messages (350).

f. Encapsidation and assembly

The unspliced and singly spliced mRNAs provide new viral genomes and also encode the structural proteins (Gag and Env), the enzymes (Pol) and the mRNAs for some of the regulatory proteins (Vif, Vpr and Vpu) which are exported to the cytoplasm and translated. Encapsidation of the viral genome is followed by budding from the plasma membrane.

Encapsidation is a process in which the products of gag and pol are incorporated into virions in the form of polyprotein precursors. Aggregation of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ at the plasma membrane via the attachment through $p17^{gag}$ (MA) initiates assembly (325). At this stage particles are seen as crescent-shaped buds. $p17^{gag}$ has a myristylated amino terminus that mediates attachment to the cell membrane (46, 140). The genomic RNA is brought to the site of assembly by its interaction with the p9 (NC) domain of the Gag precursor. The essential cis-acting sequences for HIV packaging are located at the 5' end of the genome. The NC domain is located at the C-terminal domain of the gag precursor. In common with other RNA binding proteins NC has a high concentration of basic amino acids. As well this protein has two zinc binding motifs, $CysX_2CysX_2HisX_4Cys$. Mutation of either

zinc finger or deletions of the basic residues near the first zinc finger cause a packaging defect (98, 139). The NC protein interacts directly with the genomic RNA at a region of the RNA known as the packaging signal or psi (ψ). Psi is deleted by all splicing events thus guaranteeing that only full length RNA will be encapsidated (200). Psi is located between the splice donor and gag but further analysis has shown that other regions of the HIV chromosome are also important for packaging (33, 49, 286).

The polyprotein precursors are cleaved by the viral protease prior to budding(175). Complete budding leads to the formation of immature particles with spherical cores. The viral particle contains two copies of genomic RNA-these two RNAs are linked together near their 5' ends by the dimer linkage structure (DLS) (10, 34, 139).

The previous two paragraphs have discussed production of free virus. It may be inferred from this detailed explanation that the vast majority of newly infected cells arise from the spread of the free virus but this is not the case as HIV spreads much more efficiently by cell-cell fusion. It is not known how much of the viral spread can be attributed to cell-cell fusion but it is thought to be an important route for the transmisson of virus and therefore contributes to its pathogenesis (44, 65).

6. HIV pathogenesis

HIV infection is characterized by three phases-initial infection, clinical latency and AIDS (201). For about 70-80% of all HIV-infected people the average time from infection to onset of disease is ten years (47, 116, 205). A significant proportion of individuals (~10%) progress to AIDS within 2 to 3 years of infection (rapid progressors) (247, 273). As many as 12% of infected individuals will remain free of AIDS for 20 years (247).

HIV is normally transmitted from person to person by genital secretions, notably semen, or less commonly blood. The first cells infected are resident tissue macrophages of the genital tract or rectum (293, 373, 387). The virus is then transported to the draining lymph nodes where it replicates extensively (115). A notable exception to this scenario is in the case of intravenous drug users. Although the virus is received directly into the blood the M-tropic strains still predominate in the early stages of infection (327).

Several weeks after infection there is an early phase with a burst of viral replication, high levels of viremia and HIV in the PBMC (70, 92, 143). This results in the dissemination of the virus to various tissues in the body. At this time an acute symptomatic syndrome similar to mononucleosis is frequently (50 -70%) (346) associated with the primary infection (70, 92, 143). The immune system responds to the invader and weeks to months after infection a humoral and cellular immune response is detected and HIV levels decrease. (41, 92, 144, 190, 241, 261, 280). Expansion of CD8+ cytotoxic T cells (CTLs), and natural killer cells occurs (41, 190, 261, 280). CD8+ cytotoxic T cells, natural killer (NK) cells and antibody dependent cell-mediated cytotoxicity (ADCC) may all contribute to virus decline by lysing infected cells, while neutralizing antibodies mop up free virions.

It was originally thought that HIV entered a true latent stage where no viral replication occurred (201). Since then techniques for detecting viral RNA reveal virus in the plasma at every stage of the disease (274). After the initial infection a chronic state known as the clinical latent phase arises. The clinical latent phase is of variable duration with a low but constant number of virus and infected cells in circulation and usually very gradually declining numbers of CD4+ T lymphocytes (260). During this time the lymphoid organs

serve as the primary site of virus replication (109, 262, 264, 274). The viral load at early times post infection can be used to predict when the infected individual will progress to a disease state of viraemia (299).

Within a year of seroconversion, each infected individual establishes his or her own "set point" of quasi-steady state levels of plasma HIV RNA (between 10^2 and 10^6 copies per milliliter of plasma) (287). During clinical latency many factors are involved in determining viral level. Viral factors include replicative fitness, mutation rate and cytopathicity (78). Host factors include cellular activation, as well as cytokines and their receptors (115). At this point in the infection the virus is turning over very rapidly (156, 362). Most of the virus is produced by newly infected CD4+ T cells (156, 271, 362). In patients with a CD4+ count of 500 or less, 10^7-10^9 virions are produced per day resulting in the destruction of ~$2X10^9$ CD4+ T cells per day (226). The persistent virus replication and the chronic immune activation associated with it are responsible for the progressive decline of lymphoid tissue and the decline of the immune system (116, 264). CD8+ T cells are thought to be important in the immune response to HIV during the latent phase of HIV infection for the elimination of productively infected cells and for control of viral load (358). The CD8+ T cells are also involved in the immunopathogenesis of HIV infection. They contribute to the depletion of antigen presenting cells by cell killing or tissue damage after the release of cytokines TNF α/β and interferon γ during cytolysis (115, 202, 263, 264).

Early in the latent period neutralizing antibodies may contribute to the control of HIV infection (187, 241). As HIV variants emerge over time, new variants are not neutralized (187, 241). After about 10 years of clinical latency the numbers of CD4+ cells decline to

very low values and the symptoms of AIDS appear, coincident with sharply increasing levels of virus and infected cells in circulation (78). Without treatment, death follows within a year of an AIDS diagnosis (202).

7. AIDS

Progression to AIDS usually occurs when the CD4+ counts are below 200 cells/μl; at this level of cells there is an increased susceptibility to opportunistic infections and neoplasm. The transition from nonsyncitial inducing to syncitial inducing HIV strains has been reported to be associated with rapid progression to HIV disease (16, 66, 188, 304, 305, 342). AIDS is associated with a generalized activation of the immune system, manifested by elevated serum concentrations of neopterin, soluble IL-2 receptor, soluble CD8+ and ß2-microglobin (261, 314, 315). As the disease progresses, both cell-mediated and humoral immune responses are severely impaired, resulting in the impaired regulation of both T and B cell functions (71-75, 144, 215)

As mentioned previously most infected persons develop AIDS ~10 years after the initial infection. The rate at which individuals progress to AIDS is multifactorial-both host and viral factors contribute to its development. Exogenous host factors contribute to the pathogenesis of AIDS (115). HIV replicates more efficiently in activated cells (14, 125, 381). Infection with other pathogens can increase the viral load and is associated with an accelerated disease progression (26). Immune activation is associated with elevated levels of HIV-inducing cytokines such as TNF-α and IL-1ß and IL-6 (45, 344).

8. Anti-viral agents

In general the difficulty in developing anti-viral drugs comes from the degree to which viruses parasitize their host cells. A virus must rely on the host cell for most of the enzymatic processes that it needs to complete its infective cycle. This makes it difficult to develop anti-viral agents that will selectively inhibit the virus without damaging the afflicted host. A detailed understanding of the molecular biology of HIV makes it possible to identify areas of the virus life cycle that are vulnerable to attack.

When the cause of AIDS was found to be a retrovirus it was known that retroviruses possessed the enzyme reverse transcriptase. As reverse transcriptase is not commonly found in eukaryotic cells it was a logical target for the development of anti-viral compounds. Nucleoside analogs were the first anti-HIV agents developed (166). In 1984 3'-azidothymidine (AZT) was identified as being active against HIV in tissue culture. Since this time other nucleoside analogs have been licensed for use including: 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC) and many others. From the beginning it was clear that the nucleoside analogs were not the answer to HIV. Although they do inhibit HIV replication, they do not completely suppress disease and each has its own associated toxicities. AZT treatment for prolonged periods results in the emergence of drug-resistant viral isolates that display normal replication kinetics and up to 100-fold resistance to the drug (192, 292). In patients where drug cocktails having as many as three reverse transcriptase inhibitors are administered drug resistance still appeared (193). It became clear from this that other antiviral targets were needed.

Beside reverse transcriptase, the virus contains a few enzymatic functions that are unique and well enough understood to be the targets of anti-retrovirals. The next enzymatic process that was targeted by an anti-retroviral was the virus protease. HIV's aspartyl protease cleaves the large Gag and Gag-Pol polyproteins into functional units (17). This protease is essential for the formation of mature virions and without its activity the virus cannot complete its infective cycle. Important to its success as an anti-viral, the aspartyl protease is distinct from human proteases. In 1988 the first protease inhibitors were identified. Although they were effective in tissue culture, problems associated with insolubility and retention made them unsuitable for human trials. By 1992 these problems had been resolved to the degree that several had been identified as suitable therapeutic agents. Saquinavir, indinavir, and nelfinavir are all peptidomimetic substrate analogs that inhibit the binding of natural substrates to the enzyme (229). In 1995 Saquinavir was the first protease inhibitor to be licensed for use. At about the time that the protease inhibitors emerged as the new generation of anti-viral drugs, it became apparent that what was important in determining the survival of an HIV- positive person was the reduction of viral burden (78).

Protease inhibitor mutants arise when sub-optimal doses of the inhibitors are used. This is consistent with the thinking that the more effective anti-virals will suppress viral replication and thereby slow the rate of viral evolution toward resistance (82). It was soon demonstrated that if these drugs were used in appropriate concentrations, they resulted in sustained virus suppression and delayed drug resistance without toxicity. This translated into increased survival for those receiving the protease inhibitors.

To date the most effective drug therapy has been the combination of zidovudine, lamivudine, and indinavir (287). In patients with advanced HIV infection the triple combination cleared the viremia to below the limits of detection by PCR (500 copies/ml). A disadvantage in using drugs to treat HIV infection is that because treatment must be life long, drugs will accumulate in the patient and the associated toxicity may outweigh the benefits. As well, the infidelity of the reverse transcriptase provides the virus with a high degree of mutability and this provides it with a possible means of escaping from anti-HIV therapies.

9. Vaccines

HIV's mutability has made the development of antiviral drugs difficult, and it has also been the major stumbling block to vaccine development, although other factors contribute to this difficulty.

1. HIV infection affects the immune system-both the virus and immune attack deplete the effector cell types.

2. An effective vaccine has to protect against transmission of free virus and virus-infected cells

3. An inappropriate immune response will increase the pathogenic effects of the virus.

4 There are no animal models for vaccine testing (39).

The conventional routes of vaccine development have not proven to yield any promising results. It is clear that the challenge of developing an HIV vaccine will challenge scientists to develop new approaches to this problem. One of these up and coming approaches is gene therapy. Gene therapy has the potential for use in both antiviral and vaccine capacities.

B. Gene Therapy

Gene therapy is defined as the transfer of new genetic material to the cells of an individual with resulting therapeutic benefit to the individual (243). Gene therapy involves the delivery to target cells of an expression cassette made up of one or more genes and the sequences controlling their expression. This can be done ex vivo, in which the cassette is transferred to cells in the laboratory and the modified cells are then administered to the recipient (246). Using in vivo means, the expression cassette can be delivered directly to cells within an individual.

Potential candidate diseases for gene therapy include inherited and acquired genetic diseases. For inherited diseases the strategy most often used involves the introduction of a gene that will compensate for a defective gene. There are currently gene therapy trials in place for adenosine deaminase deficiency, Gaucher disease, familial hypercholesterolemia, muscular dystrophy, emphysema, hemophilia B, Fanconis anemia and cystic fibrosis (196, 246). These genetic diseases are considered to be best suited and justified for gene therapy as there is a single recessive gene mutation, the natural history is either fatal or crippling, and conventional therapies are inadequate (243). The isolation and cloning of the gene responsible for the disorder was another important determinant for its selection as a suitable candidate for gene therapy (243).

Acquired genetic disorders include cancer and AIDS. The majority of approved human gene therapy clinical applications that have been directed towards the treatment of cancer are the incorporation of suicide genes and immune boosting strategies (243).

HIV is a suitable target for gene therapy because a normal part of the HIV replicative is to integrate the provirus into the target cell chromosome. The virus remains as a heritable feature of the cell and in this sense AIDS is an acquired genetic disorder.

Most gene therapy approaches involve a vehicle or vector for the delivery of foreign DNA. The vectors employed are commonly retrovirally derived (87). Retroviral vectors provide the distinct advantage of integration of the foreign gene. Retroviral vectors do not contain any native viral protein, thus limiting undesirable immune responses (354).

1. Retroviral Vectors and Packaging cell lines

Retroviral gene therapy systems consist of two components, the retroviral vector and the packaging cell lines (8, 222, 359). Vectors are deleted versions of the virus they are based on; they have had their structural genes removed and replaced with therapeutic and/or marker genes (167, 234, 237). The vector is manipulated in it's DNA form as part of a bacterial plasmid. Most retroviral vectors are based on Moloney murine leukemia virus (MLV) but they are not exclusively limited to this and there are even retroviral vectors based on HIV (251). MLV is a naturally occurring retrovirus that causes T cell lymphomas in susceptible mice after a relatively long latency (243). Retroviral vectors contain only the viral nucleic acid sequences required in cis. To be useful in therapeutic applications the vector must be replication defective but able to direct reverse transcription, integration and RNA transcription (354). Vectors usually contain a 5' and 3' LTR, a packaging sequence and a transcription unit. The LTRs may or may not be essential to direct the synthesis of the therapeutic or marker genes. LTRs are essential to the vector as they contain the primer

binding site and purine rich sequences that are necessary for reverse transcription. The LTRs also have specific sequences near their ends that are necessary for integration (259).

As it is already present in the vector, the retroviral LTR is often used as the expression cassette for the therapeutic or marker genes (354). The LTR can be modified to influence expression or to respond to certain stimuli (245, 290).

The vector needs to be packaged in the form of an infectious particle. All the structural proteins needed to allow production of infectious virions are supplied in trans by a packaging cell line (354). The helper functions are stably expressed in packaging cells made by the transfection of plasmids containing the coding sequence from one or more replication incompetent provirus. The RNA produced from the integrated sequences is not packaged because it lacks the packaging signal required for virion production. As the therapeutic virus is essentially produced from two separate components, it is relatively easy to introduce foreign proteins into the virion for pseudotyping and retrovirus stability (8, 23, 84, 85, 300).

The packaging cell line is made with several aims in mind; to produce a therapeutic virus that contains an RNA version of the gene therapy vector, to produce large amounts of recombinant virus and to avoid the breakout of replication-competent virus. Another consideration in the choice of a packaging cell line is the desired host range of the vectors. Host range is determined by the packaging cell line. Murine type C retroviruses can be classified according to receptor utilization, amphotropic, MLV-A, (allows infection of most mammalian cells), ecotropic, MLV-E, (viruses can only infect murine cells) and xenotropic MLV-X (can infect cells that are not murine derived). Tropism is determined primarily by

the binding interaction between the viral envelope glycoproteins and specific proteins on the host cell surface that act as viral receptors. The MLV-E receptor is a cationic amino acid transporter (9); the receptor for MLV-A is an inorganic phosphate transporter (239).

Packaging cell lines are produced by transfecting a plasmid encoding gag, pol and env and selecting for cells that express these proteins and can support vector production (206, 233). The plasmid is engineered with the minimum sequence information necessary to produce viral proteins. This is done to avoid recombination between the vector and the packaging cell line. Several strategies have been used to minimize recombination; the 3'LTR is commonly removed and replaced with a generic polyadenylation signal (104), the LTR can be modified either by deletion of inclusion or an exogenous promoter (237), or the viral genome can be split into two transcription units (224).

2. Specific problems of gene therapy for HIV

The following constraints apply to the choice of target tissue for MLV retroviral delivery of anti-viral genes. Included is cell division. MLV vectors only infect actively dividing cells. HIV can infect non-dividing cells, therefore the target of the therapy and the therapeutic element may not connect. There are at least two solutions to this: One is to create a gene therapy vector based on HIV. As the vector is composed of HIV proteins it would have the same tropism as the originating virus (i.e. HIV) (251). The other is to target cells that are early enough in the hemopoeitic lineage that all arising cell types carry the protective gene (354).

Also inhibiting appropriate targeting is the disseminated nature of HIV infection. The central nervous system is almost always involved; perivascular macrophages and microglia

are the primary reservoirs of HIV (201). The blood brain barrier makes this area of the body very difficult to target. There is some hope generated by the observation that extensive viral replication in the brain only occurs in the late stages of HIV infection. Therefore an effective therapy that is administered at early times may eliminate the need for direct treatment of the CNS.

Another difficulty associated with HIV treatment is that HIV behaves as an autosomal dominant. In the treatment of recessive disorders the replacement of a lost gene function is enough to guarantee some improvement (243). The task of HIV treatment is made more difficult because simple gene addition is not going to be good enough; the inserted gene must be able to suppress the product of the virus in a competitive manner.

3. Limitations of MLV Based Vectors

The MLV system is limited by the titer of the virus that can be produced. Retroviral vectors have a relatively short half life (3.5-6.5 hr at 37 C depending on the vector constructs) (268). The upper limit of virus titer produced in culture is in the range of 10^7 particles per ml. It has been shown that the titer can be increased by centrifugation (189) or pseudotyping with VSV G protein (8).

Size of the retroviral genome is a further limit to the MLV system. The upper limit of DNA that can be incorporated into an MLV based gene therapy vector is 7 kb (236).

Non-specific integration of the retroviral construct has associated difficulties. Aside from the small risk of insertional mutagenesis, variability of expression is the major problem arising from lack of site-specific retrovirus integration.

Stability of gene expression. Cells transduced with retroviral vectors ex vivo and then returned in vivo often lose expression of the transduced gene over a short period, this may be due to methylation (258, 285) or deletion of the proviral DNA (298). For example murine pluripotent hematopoietic stem cells were assayed for MLV driven expression. In this system long term expression did not occur and this was shown to be due to methylation (59).

4. Potential strategies for gene therapy of HIV infection

a. Protein based therapies-Trans-dominant anti-HIV proteins

One of the first suggested applications for gene therapy was the in vivo expression of mutant forms of viral proteins that can interfere with the function of the wild type version. This effect may be due to inhibition of normal function or disrupting assembly of a viral protein complex. This is known as a trans-dominant negative phenomenon, wherein a relatively small number of mutant proteins can disrupt a larger number of wild-type proteins. Trans dominants for Rev (216, 219, 230), Tat (145, 240), Env (48, 108), Gag (349) and the protease cleavage site (17) have been made.

Efficient Tat mutants appear to act by sequestering cellular factors involved in viral transactivation. These mutations occur in the RNA binding/nuclear localization domains of Tat (145, 240). Mutations which either delete the HIV-1 or HIV-2 Tat basic domain or alter the charge by substitution of neutral amino acids result in proteins that are very defective in activating HIV gene expression (100, 129, 296, 297). These result in transdominant proteins which inhibit wild-type Tat activation of gene expression (107, 240, 270).

Rev mutants appear to compete with the wild-type protein for binding to the RRE (221). The most extensively studied of these is the RevM10 mutant. It has mutations in the leucine-rich domain close to the protein's C terminus (216, 219). The leucine mutants bind to the RRE and multimerize but fail to interact with cellular factors involved in RNA transport. Rev contains an arginine-rich domain necessary for oligomerization and nuclear localization (4, 159, 216, 219) and an activation domain which contains the critical leucine (4, 159, 216, 219). RevM10 inhibits wild-type Rev function and when it is expressed in T-cell lines and PBMC HIV replication is reduced (19, 35, 218).

Gag derived transdominant mutants act by forming non-functional multimers and interfere with the generation of infectious particles (349). The Env mutant 41.2 acts by interfering with the binding of env to the CD4 receptor (48, 108). The 41.2 mutant has a substitution in the second amino acid of gp41.

Limitations of transdominant proteins: events are confined to post integration events, the resulting immune response may destroy the transgene-expressing cell and some of the expressed proteins interact strongly with nucleic acids sequences and may disrupt normal cellular function.

b. Other protein based strategies

Other types of protein based strategies include targeting cellular factors, suicide genes and single chain antibodies. Exogenous recombinant sCD4 can block HIV-1 infection in vivo (96, 124, 162, 322, 360). Single-chain variable fragments (SFvs) can be derived from the antigen-binding region of antibodies, and stably expressed in cells. SFvs represent the variable light (Vl) and variable heavy (Vh) chains of monoclonal antibodies, bound together

by a flexible linker peptide (368, 372). These constructs can be efficiently expressed intracellularly (37). The variable regions of monoclonal antibodies have been shown to contain the antigen-binding site of the parent antibody (25). An intracellular antibody to Rev has been reported to inhibit HIV in human T-lymphoid cells (310). Genes for the production of such toxic proteins as herpes simplex thymidine kinase (55, 352) and diphtheria toxin (99) are introduced into the cells. To make them inducible these genes are placed under the control of the HIV-LTR and only when Tat is present are they transcribed in high levels.

c. Drawbacks of protein based therapies

Protein based therapies may lead to the induction of an immune response directed against the gene-modified cells (288, 379). Trans-dominant proteins may increase the virus-related cytopathicity as was seen in the case of a trans-dominant for gag that caused increased cell killing. Also trans-dominant proteins are targeted to post integration events.

d. Non protein based therapy

RNA based strategies are less likely to induce immune responses to the genetically modified cells. There are three classes of RNA-targeted antiviral agents: antisense (DNA and RNA), ribozymes and RNA decoys.

Antisense RNAs are naturally produced by prokaryotic cells and bacteriophages for the modulation of gene expression. Antisense RNA recognize and bind target RNA sequences and they can be used in anti-viral applications as they block viral replication by competitive hybridization. The RNA-RNA duplex that is formed is subsequently degraded (295).

Intracellular expression of anti-sense RNAs is considered to be a potential means of antiviral suppressive gene therapy. Antisense nucleic acids have been used successfully as inhibitors of gene expression and viral replication in prokaryotic and eukaryotic cells (295). HIV- targeted genes have been developed against transcripts coding for Tat, Rev and Vpu (281), the leader gag region (308, 353), reverse transcriptase (232), primer binding site (170) and the packaging signal of HIV (81). Anti-Tat tRNA constructs have also been used; the inclusion of the tRNA is advantageous as the transcript is produced by RNA polIII and the antisense sequence has the stability and intracellular fate of a tRNA. PolIII transcription takes place in all cell types with no evidence of promoter inactivation through methylation (36). Therefore any transgene that was driven by a PolIII promoter would be expressed constitutively and without cellular bias. Veres et al demonstrate that the length of the anti-sense transcript is important to its efficacy and longer transcripts appear to be more effective in suppressing HIV replication (353).

Antisense requires at least a 1:1 ratio of antisense and HIV-1 target RNAs. Lo et al showed that the state of protection provided by an anti-Tat construct could be overcome by the build-up of RNA (209).

Ribozymes are an extension of antisense technology. They are small RNA molecules catalyze sequence-specific RNA cleavage reactions (56). Ribozymes identify their target sequences through antisense binding and after binding cleave the target. They are more efficient than strictly antisense molecules because they are reusable and theoretically do not need to be present in greater than equimolar amounts. Hammerhead ribozymes are among the best characterized of the catalytic RNA molecules; they cleave RNA in trans via a small

conserved catalytic center that recognizes the trinucleotide sequence GUX (X=A,U,C) and possess two flanking antisense sequences that direct the ribozyme catalytic center to substrate RNA through base pairing (149). A hammerhead ribozyme was the first to be developed as a therapeutic agent in vitro (301). Hammerhead ribozymes have been targeted to a number of HIV transcripts: Tat (86), gag (301) vif (210) integrase (319) and the leader sequence (361). The hairpin ribozyme is produced by the tobacco ringspot virus; it can be used to target exogenous RNA according to the following rules. The ribozyme recognizes a BN*GUC sequence in the target. The ribozyme base pairs to either side of the target sequence and cleaves the RNA at the site shown by the astrix, where N is any nucleotide and B is U, C or G. Yu et al. used the hairpin to attack the 5' leader of HIV (380). Other groups have also targeted the LTR (195) and rev/env sites of HIV (378).

This work has given insight into several parameters that this therapy must overcome. The ribozyme must be delivered in high enough amounts, to be effective and it must anneal to an accessible region of the target mRNA.

e. RNA decoys

RNA decoys are short RNA oligonucleotides which mimic critical regulatory sequences on HIV RNA. Decoy elements for HIV TAR (207, 337), HIV RRE (197, 198) and the HIV packaging signal (31) have been created. TAR and RRE decoys inhibit HIV expression and replication by competing with the authentic elements for Tat and Rev binding. The RNA decoy target sequences are highly conserved among HIV-1. A specific concern associated with the use of TAR or RRE decoys is that cellular factors associate with these structures and consequently may be sequestered in cells over-expressing decoys. An

interesting solution to this is to express a TAR decoy using a promoter that contains the HIV TAR element (207). TAR would only be expressed in high levels in cells containing HIV Tat.

f. HIV packaging signal as a decoy RNA

Much of this thesis deals with the creation and testing of a decoy packaging signal (Chapter 3). As previously introduced in this chapter a retroviral packaging signal is a cis acting sequence that appears on the unspliced viral genome. Virus particles are composed of the viral genome, virally encoded protein and host factors. In a virally infected cell, there is a mechanism by which viral protein can identify the nascent viral genome. Viral protein recognizes part of the genome based on a region of secondary structure known as the packaging signal. In HIV, the nucleocapsid protein binds to the HIV packaging signal to initiate virus assembly (10, 34, 76).

Some confusion exists as to exactly which portion of the HIV genome is the packaging signal. Deletion of the sequence between the major splice donor site and the gag coding region has been shown to decrease the encapsidation efficiency of HIV-1 genomic RNA (200). Subsequent genetic analysis established that this region serves as a cis-acting packaging signal (ψ) (10, 200). Studies of the HIV-1 packaging signal show that the ψ site alone is not sufficient for efficient packaging. Other regions in the HIV-1 genome are important for efficient encapsidation. These include regions downstream of the *gag* start codon, additional cis-acting sequences in the 5' untranslated region (UTR) and sequences in the *gag* and *env* open reading frames (48, 150, 177, 181, 200, 212, 277, 286). Successful encapsidation of HIV RNA relies on these regions, nevertheless the successful encapsidation

of HIV-1-based vectors containing only the conserved ψ locus in the context of the full length 5' untranslated leader sequences has been demonstrated (48, 150, 251, 267, 277). This last point was the reason we chose to create a packaging element based on sequences in the 5' untranslated and early gag regions. Details of that area are shown in Figure 11a. The putative packaging element described in chapter 3 contained the conserved ψ locus and sequences upstream of the splice donor site, the dimerization initiation site (DIS) (248, 321), and gag sequences. Two elements were created based on the HIV-1 packaging signal, LJC140 and PAK 140.

One of the effects that one might expect a packaging signal decoy to have is to enhance other RNA based anti-viral elements such as antisense RNA through the phenomenon of co-localization (335, 336). Co-tracking may be critical because in eukaryotic cells many RNAs do not appear to diffuse freely. Instead, they may be highly compartmentalized and actively sorted to specific cellular locations. Such RNA trafficking may result in localization of substrate RNAs in a different compartment than antisense elements, which would effectively reduce substrate RNA availability to antisense and therefore limit the effectiveness of antisense sequences as gene inhibitors (335). Theoretically including the HIV packaging signal on an RNA would allow that RNA to end up in the same compartments of the nucleus as genomic HIV. Therefore, any second anti-HIV element included on the vector, such as an antisense sequence, would be more likely to encounter its target. The "co-tracking" of the MLV vector and the HIV-1 genome may interfere with the normal function of HIV-1 during replication (336).

With the idea that two anti-HIV elements would be more effective, and the hope that co-tracking may increase their efficacy, vectors were created containing LJC140 and antisense to gag sequences as well as PAK140 and an antisense to gag sequence.

5. Goals of this study

Most of the current human gene therapy protocols make use of amphotropic MLV based vectors. Genes cloned under the control of the MLV LTR enhancer/promoter are expressed at relatively low levels in human cells, and the levels of expression vary widely between different cell types. To overcome this difficulty this study has focused on improving the MLV LTR by incorporating a foreign enhancer/promoter in this region. Modifications of this type could affect retroviral functions such as transcription, reverse transcription, packaging and integration. Thus the goal of this study was to derive a completely functional retroviral vector which would give high level expression of genes placed under control of the LTR in cells that are relevant targets of HIV gene therapy applications. The LTR should be constructed so that it would be up-regulated by the HIV Tat protein, so that expression would be greatest in cells which were already infected by, or in the process of being infected by HIV.

The results demonstrate that the MLV LTR can be modified to increase its transcription in T lymphocytes and that the vector would respond to HIV. This vector was then used to deliver the anti-viral element to cells that were subsequently challenged with HIV. The anti-viral element was based on HIV's packaging signal and has an antisense to gag section. This element was far more effective in inhibiting HIV infection when the vector used was the one with the modified LTR.

This work demonstrates that vector design is an important consideration in gene therapy applications. In general, gene therapy vectors that are specifically designed to function in relevant cell types will be far more effective in delivering therapeutic genes.

CHAPTER 2 - MODIFIED RETROVIRAL VECTOR

A. Introduction

Most gene therapy protocols that have been approved in North America use retroviral based gene therapy vectors. These vectors have a number of features that make them attractive for gene therapy applications namely; a wide range of host cells as well as permanent integration into the host cell chromosome. The science of gene therapy is relatively young and early experiments have demonstrated some shortcomings of the retroviral based vectors. In vivo applications have shown that the heterologous gene product is only expressed at low levels and not for long periods. Retroviral vectors are handicapped by their inability to infect non-dividing cells and by the current difficulties in producing high-titre recombinant retroviral stocks. These limitations inhibit general applicability of these vectors and hamper their usefulness for anti-HIV applications.

This section deals with the alteration of an existing gene therapy vector, pLNL6 (237), to produce a vector specifically designed for anti-HIV applications. pLNL6 is a gene therapy vector based on the Moloney murine leukemia virus. Ideally this vector should express at high levels in the cell types infected by HIV. Vector expression in these cells should be stable for a reasonable period and finally it should be possible to produce this vector in high enough titer to be useful for gene therapy applications. Although improvements were made in this vector with the specific aim of HIV gene therapy it was expected that the vector changes would make it more suitable for gene therapy approaches in general.

B. Methods

Restriction enzymes were purchased from New England Biolabs (NEB Mississauga, Ont, Can), GIBCO (Burlington, Ont, Can), Promega (Edmonton, AB,Can) or Boehringer Mannheim (Laval, Que, Can). T4 DNA ligase was obtained from NEB and calf intestinal phosphatase form Promega. Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA) and GIBCO. Geneticin (G418) came from GIBCO, and L-glutamine, penicillin and streptomycin, Dulbecco's minimal eagle medium (DMEM), and RPMI-1640 medium were purchased from Gibco.

1. Plasmid construction

To aid this study a large number of clones were created. Some were made by the thesis candidate and many were cloned by Dr. L-J. Chang (LJC). To avoid confusion and to give credit where it is due a map of each of the constructs used is shown in Appendix 1. Included in the map is the name of the individual responsible for making the clone.

CMV *Tat* (pCEP-*Tat*), RSV *Tat* (pREP-*Tat*), PLLL*gptSVTat*, pMT and pMCT as used in this thesis work were cloned by L. Chang. pMTcat and pMCTcat pLLL, pLLLSV, pLCTSN and pLGCTSN were also cloned by L. Chang (LJC).

pLSNcat, and pLCSTNcat and pLGCSTNcat were all made from the originating vectors in the same way. A fragment of DNA containing the cat gene was removed from the plasmid pU3Cat by cutting at the *Bam*HI and *Sau*3AI sites. This 540 basepair fragment was cloned in to the *Bam*HI site of the three gene therapy vectors.

2. Cell lines and viral strains-(Table 1)

HeLa, HepG2, Huh7 and PA317 were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS). AA-2 cells were grown in RPMI 1640 containing 10% FBS, 1X non-essential amino acids and 1mM sodium pyruvate. All cultures but PA317 were supplemented with 5% penicillin and streptomycin and maintained in a 5% carbon dioxide incubator at 37 °C. PA317 were maintained in a 10% CO_2 environment. H9 is a single cell clone derived from the HUT 78 cell line. AA-2 is derived from the WIL-2 human splenic EBV+ B-lymphoblastoid line. Both H9 and AA-2 are permissive for HIV-1 infection and are sensitive to viral cytopathic effects. H9 and AA-2, were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

3. Transfection with CaPO4 (adherent cells)

Hela cells were maintained in DMEM with 10% fetal bovine serum (FBS) and transfected by a modified $Ca_3(PO_4)_2$-DNA coprecipitation method (142). Twenty-four hours prior to transfection cells are passaged and are seeded to yield ~ 80-90 % confluency/well on a six well plate (Falcon 3046, Becton and Dickenson, Mississauga, Ont) in a final volume of 2 ml. The following recipe is suitable for transfection into one well of a six well plate. DNA (2-10 µg) was added to 90 µl of water and 10 µl of 2.5 M $CaCl_2$ (Mallinckrodt, Paris, Kentucky) and vortexed. 100 µl of 2X BBS, pH 6.95 (50mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; BES) (Sigma, Mississauga), 280 mM NaCl, 1.5 mM Na_2HPO_4 (pH 6.95) was added dropwise to the DNA and the mixture was mixed by gentle agitation after each drop. The DNA and BBS were incubated for 45 min at room temperature. At this time the cells were fed with a fresh 2 ml of media. The calcium

TABLE 1. Cell lines

Cell line	Source	Media	CD4+/-
Hela	Cervical carcinoma	DMEM*	negative
Huh7(250)	hepatocellular carcinoma	DMEM	negative
HepG2(2)	hepatocellular carcinoma	DMEM	negative
PA317(235)	NIH 3T3 mouse fibroblast	DMEM	negative
Cem	T cell lymphoma	RPMI†1640	positive
Cem TART(64)	T cell lymphoma	RPMI 1640	positive
H9 (276)	T cell lymphoma	RPMI 1640	positive
C8166(323)	T cell lymphoma	RPMI 1640	positive
MT4(192)	T cell lymphoma	RPMI 1640	positive
AA-2(58)	B cell lymphoblastoma	RPMI++1640	positive
Molt-4 (180)	T cell lymphoma	RPMI 1640	positive

* Dulbecco's modified Eagles medium with 10% fetal calf serum.

†RPMI++1640- contains 1X non-essential amino acids and 1mM sodium pyruvate.

phosphate-DNA solution was added dropwise onto the cells while swirling the plate. Cells were incubated for at 37°C in a 3% CO_2 incubator. Twenty-four hours after transfection, the cells were washed and fed with 2 ml of fresh media. All transfection experiments included a human growth hormone control plasmid for normalization of transfection efficiency.

4. Electroporation

For electroporation plasmid DNA (10 μg) was added to 10 x 10^6 cells/0.4 ml (in growth medium) in a 0.4-cm cuvette and electroporated at 250 μF, 300 V (for AA-2 cells) or at 960 μF, 300 V(for CEM, CEM-TART and H9 cells). The cells were then transferred to a T25 flask containing 10 ml of growth medium with 20% FBS.

5. Production of retroviral vectors

This thesis work was done with viruses produced from the retroviral packaging cell line, PA317 (235). PA317 were transfected using Lipofectamine (Gibco) to produce infectious virus. One day before the transfection to produce virus, 4 x 10^5 PA317 cells are seeded into a 25 mm tissue culture plate and fed with 5 ml of media. The PA317 cells are transfected using lipofectamine (Life Technology, Bethesda, MD, USA). The transfection solutions are prepared in sterile 12 x 75-mm polystyrene tubes,. 1-3 μg of DNA is added to 200 μl of serum free DMEM. At the same time 0.5 to 6 μl of lipofectamine is added to 200 μl of serum free media. The two solutions are combined mixed gently and incubated at room temperature for 45 min. At this time the cells are rinsed with serum free media. An additional 1.6 ml of serum free DMEM is added to the DNA-liposome mixture before adding to the cells. The resulting solution is mixed gently and added to the rinsed cells. Cells are

incubated for 5 hr at 37°C in a 5% CO_2 incubator, after which, 2 ml of growth media containing 20% fetal bovine serum is added. 24 hr following the start of transfection, medium was replaced with fresh. From 48-72 hours after transfection high titer virus was harvested from the culture. Virus was harvested by withdrawing the culture media and filtering through a 0.45-μm filter (Millex-HV, Millipore, Bedford, MA, USA) or alternatively by spinning the culture for 5 min at 3000 g. The filtered or centrifuged culture was frozen on dry ice in screw cap eppendorf tubes and stored at -80°C.

6. Generation of producer cell lines

To create cell lines that stably produce therapeutic virus, cells from a transiently transfected preparation were passaged to yield a low level of confluency (~10%) and placed under selection. All of the gene therapy vectors used in this study had the *neo* selectable marker. The concentration of G418 used to select producer cell lines was 200 μg/ml and producer cell lines were obtained approximately 10 days after the start of selection.

7. Infection with virus produced from packaging cells

Susceptible cell types can be infected by exposure to virus. Infection can be facilitated through the addition of polybrene (77) to the infecting virus mixture. Infection of adherent cells is done by adding the virus in a reduced volume of media-generaily 1/4 of the media usually used for normal feeding is used and is applied to the cells for 3 hours. Cells are plated at a low enough density to allow cell division during infection.

8. Titering virus produced from transfected packaging cell lines

To determine the packaging efficiency of modified vectors, the packaging cell line PA317 was transfected with each vector using lipofectamine. Virus containing supernatant

was harvested from the transfected cell lines after 48 h. The recombinant viruses were titrated by retroviral transduction of Hela or HepG2 cells. Hela/HepG2 were split into six-well plates at 5 x 10^5 per well 17-20 h before infection. Viral stocks were used at dilutions of 1:100, 1:1000, 1:10,000 and added to each well in 500 µl of growth medium containing 4 µg/ml polybrene. After 2 h incubation the plate was fed with 2 ml of complete medium and 24 h later the culture was split 1:20 and plated in selective medium. G418 resistant colonies were counted about 8-10 days later by coomasie brilliant blue G staining (1 g/l in 40% methanol and 10% acetic acid).

For long term expression studies, cell colonies were pooled or single cell clones picked after 2 weeks of G418 selection using a cotton swab soaked with trypsin. The cells harvested on the cotton swab were replated, maintained under selection for 1-2 months, and then used to prepare cell lysates for the CAT assay. CAT activity was normalized to the total quantity of protein in each lysate.

9. PEG fusion

An equal ratio of two different cell lines was plated into one T25 flask at approximately 90% confluency 24 h before poly-ethylene-glycol (PEG) fusion (PEG 1500, Boehringer Mannheim, Indianapolis, IN. USA). The cells were washed 2 to 3 times with phosphate buffered saline (PBS) and 1.5 to 2 ml of PEG 1500 was added to the cells for a 5-min incubation at room temperature with occasional tilting. The cells were then washed with growth medium three to four times to remove residual PEG and fed with 5 ml of growth medium. The CAT assay was preformed 48 h after PEG fusion.

10. CAT assay

Chloramphenicol acetyl transferase (CAT) inactivates chloramphenicol by the formation of mono-and diacetylated derivatives (312). The assay used to measure this activity is based on one described by Shaw and Brodsky (313). Cell extracts were made 72 h after transfection. Prior to lysing the cells were washed 3 times with PBS. One ml TEN buffer consisting of 50mM Tris-hydrochloride (pH 7.5), 1mM EDTA and 100 mM NaCl, was added to the cells and incubated for 10 min. Adherent cells were removed from the flask with a rubber policeman. The cells were pelleted in an Eppendorf microfuge by spinning at 5000 RPM for 2 min. The cell pellet was resuspended in 100 µl of 0.25 M Tris-hydrochloride (pH 7.8). Cells were lysed by three cycles of freezing and thawing. The supernatant was removed and assayed for enzyme activity. The assay mixture contained (in a final volume of 145 µl) 95 µl of 1 M Tris-Cl (pH 7.8), 30 µl of cell extract, 1 µCi of (^{14}C) choramphenicol (50 µCi/mmol; Amersham), and 20 µl of 4 mM acetyl coenzyme A. The cell extract and Tris-Cl components were varied if the reaction did not fall in the linear kinetic range; that is a detectable signal within 1 h and less than 50% consumption of substrate. The components are incubated at 37°C for 40 min to 2 h. The reaction was stopped by adding 1 ml of cold ethyl acetate, which was also used to extract the chloramphenicol. The sample was dried by evaporation on a Savant speed vac. The residue was resuspended in 30 µl of ethyl acetate, spotted on polyester backed silica gel thin-layer plates (Fisher) and run with chloroform:methanol (19:1). The plates were analyzed using a phosphoimager (BAS1000; Fuji, Stamford, CT, USA).

11. Northern analysis and RNase mapping

Cytoplasmic polyA+ RNA was harvested essentially as described (61). Cells were washed with ice cold PBS and resuspended in 250 µl of solution 1 (10 mM Tris-HCl pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$, all RNase free) containing 25 µl of 40 mM VRC (vanadyl ribonucleoside complex, Life Technology). After incubating 5 min on ice, 12.5 µl of 10% NP40 was added (final concentration (Cf) 0.5%), and the tube was vortexed and spun in a microfuge at 1310 *g* for 3 min. The supernatant (250 µl) was transferred into a second tube containing an equal volume of 2 times proteinase K buffer (200 mM Tris-HCl pH 7.5, 40 mM ethylene diamine tetra-acetic acid (EDTA), 300 mM NaCl, 1% sodium dodecyl sulfate (SDS) and 0.2 mg/ml proteinase K), mixed, and incubated at 37°C for 30-60 min. PolyA+ RNA was isolated by adding NaCl to Cf 0.5 M, SDS to Cf 1%, and 30 µl of dry oligo-dT cellulose powder (Collaborative Research, Bedford, MA, USA), and rotating the tube at room temperature for 1 h. The oligo-dT cellulose was then pelleted and washed twice with 650 µl of high-TEN-SDS (20 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5 M NaCl, 1% SDS) and one time with 650 µl of low TEN-SDS (0.1 M NaCl instead of 0.5 M). The polyA+ RNA was then eluted in two 200-µl aliquots of TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and precipitated by adding 40 µl of 5 M ammonium acetate and 1 ml of 95% ethanol. The RNA blot was made using standard methods and hybridized to a ^{32}P-labelled *cat* gene probe. For normalization, the blot was stripped and rehybridized with a β-actin probe.

RNase mapping was performed as described (61). The probe was made using pU3CMV5'CAT which contains the *Nhe*I to *Eco*RI fragment (961 bp) of pMCT-cat. The plasmid was linearized by *Sal*I and transcribed with T7 polymerase according to the

manufacturer's protocol (Promega, Madison, WI, USA). DNA template was removed by DNase digestion and the α^{32}P-UTP (NEN) labeled RNA was resuspended in formamide and stored at -20°C. RNase mapping was performed by mixing the polyA+ RNA with the riboprobe in a total volume of 30 µl of 1 times hybridization buffer, 80% formamide (5X hybridization buffer: 2 M NaCl, 5 mM EDTA, 0.2 M MOPS 3-morpholino-propanesulfonic acid pH 7.0), incubating 90°C for 5 min and then 40°C overnight. To degrade unhybridized RNA, 30 µl of RNase digestion buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.3 M NaCl, 40 mg/ml RNase A, 2 mg/ml RNase T1, Sigma) was added and the mixture incubated at 30°C for 1 h. After RNase digestion, 10 µl of 20% SDS and 5 µl of proteinase K (10 mg/ml) were added and the mixture incubated at 37°C for 15 min to destroy residual RNases. The final product was extracted once with phenol-chloroform and precipitated with 2.5 volumes of ethanol. The RNA hybrids were resolved on a 6%-non-denaturing polyacrylamide gel. The radioactive signals were analyzed by autoradiography and quantified using a phosphoimager (Fuji, BAS1000).

C. Results

1. Activity of MLV promoter with other viral enhancer/promoters

As most human gene therapy protocols use the amphotrophic MLV based vectors, we chose this system as the starting material for the anti-HIV gene therapy vector. The MLV system is not ideal for HIV gene therapy applications because genes cloned under the control of the MLV LTR are expressed at low levels in human cells. To enhance expression we attempted to improve the MLV LTR by incorporating a stronger enhancer/promoter into this region. To identify a promoter that would have a high function in the appropriate cell types

we compared the promoter activity of MLV LTR, HIV LTR activated by Tat (pCEPTat is cotransfected to provide Tat to this system) and CMV-IE enhancer/promoter (Figure 4). The last two promoters have been shown to be highly transcribed in a variety of cell lines. To measure their relative activity the promoters were placed upstream of a *cat* reporter gene, transfected into Hela cells and after 2 days cell lysates were prepared and assayed for CAT activity. The MLV promoter is at least 10-fold less active than either CMV-IE or HIV-1 LTR (Tat).

In most gene therapy constructs, however, an internal promoter rather than the MLV LTR has been used to drive expression of the desired heterologous gene. One of the most frequently used internal promoters has been the SV40 immediate-early enhancer/promoter. Using a slightly different assay system (transactivation of an HIV LTR which in turn drives CAT expression) we compared a series of four different promoters including; (1) the SV40 promoter by itself; (2) the SV40 promoter internal to a MoMLV LTR; (3) the CMV-IE promoter; and (4) the Rous Sarcoma virus (RSV) LTR promoter. Each of the four promoters was cloned upstream of the gene encoding HIV Tat, and plasmids were cotransfected into HeLa cells along with a second containing the CAT gene driven by an HIV-1 LTR. In this case transactivation of CAT expression is used to measure promoter strength, and this is compared with levels of CAT expression which occur with the HIV LTR/CAT plasmid alone (*X-tat*-pA, the no promoter control, Figure 4b). Not unexpectedly, the CMV promoter was determined to be considerably more active than the SV40 promoter (SV40 alone or inserted within the MLV promoter). In addition, the SV40 promoter by itself is two times more active than the 'internal' SV40 promoter (MLV/SV40).

The activity of these promoters were compared in HeLa cells (Figure 4). It is clear from these results that the MLV promoter is not very active in cell types relevant for general and HIV directed gene therapy. Either CMV-IE or the activated HIV-LTR may produce higher levels of expression. On the basis of these data we chose to incorporate elements of these two strong promoters into LNL6.

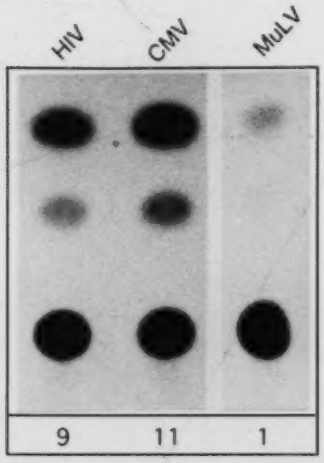
2. Reconstruction of the MLV LTR and testing of the hybrid promoter constructs

In another study (62) it was established that the HIV LTR could be modified with the inclusion of CMV-IE enhancer/promoter elements; this chimeric promoter has a high basal level of activity but could still be upregulated by Tat. Dr. Chang therefore chose to reconstruct the MLV promoter in a similar way by including a truncated CMV-IE enhancer element known as dl.CMV-IE and the HIV-1 TAR in the MLV LTR. Dr. Chang inserted these elements into the 3' LTR of the retroviral vector pLNL6 (Figure 5). The dl.CMV-IE element was not a complete promoter and consisted of a subset of the upstream enhancer elements (62). pLNL6 contains a MLV LTR at the 3' end of the provirus and a murine sarcoma virus (MSV) LTR in the 5' end. After production of infectious therapeutic virus the 3' promoter elements are used to drive the gene of interest. As discussed in the introduction, promoter elements in the 5' LTR are lost during the retroviral replication process.

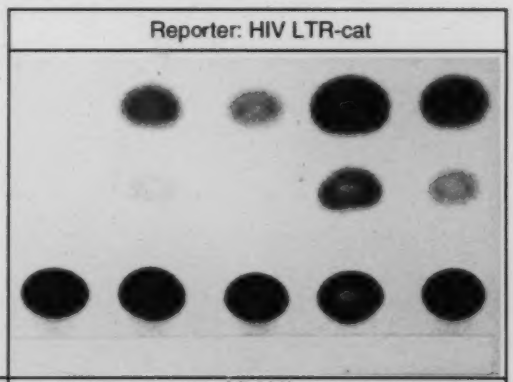
Plasmids constructed in this way cannot be assessed by transfection assays. To directly determine the effects of the modifications on promoter strength, plasmids were constructed that contained the modified LTR sequences that drive the expression of the reporter gene CAT. Before modifications of the LTR, these plasmids contained the 3' LTR

FIGURE 4. Comparison of heterologous promoter activities with the Moloney MuLV LTR. (a) Relative promoter strengths of HIV, CMV-IE and MuLV LTR in HeLa cells. HeLa cells were simultaneously transfected with a human growth hormone plasmid as an internal control for transfection efficiency. CAT activities were determined 48 h after transfection, and the relative levels of expression are shown. (b) Study of SV40 promoter activities internal and external to MuLV LTR by Tat transactivation assay. This assay is described in the text. The HIV-1/LTR-cat (pU3-R-CAT) reporter plasmid was transfected along with one of the tat expressing plasmids. CAT activities reflected the levels of Tat expressed from the heterologous promoters (SV40, MuLV/SV40, CMV or RSV). Figure prepared by LJC.

Relative Promoter Strength in HeLa Cells



Promoter Activities Determined by Function of Tat Trans-activation



Promoter	X	SV40	MuLV/ SV40	CMV	RSV
%Conversion	0.2	10.3	5.4	66.2	28.3

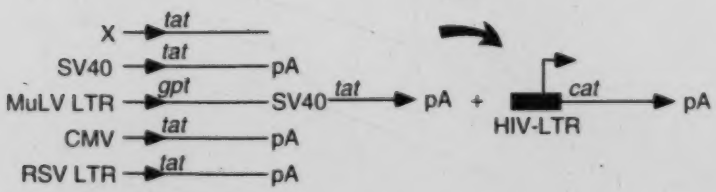
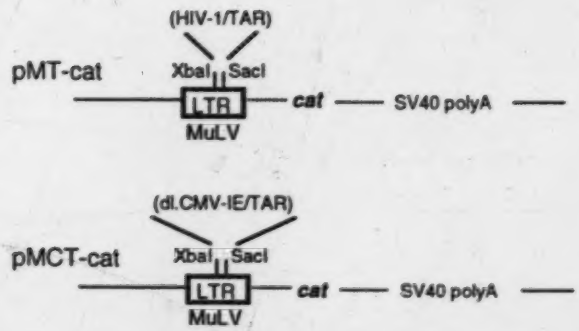
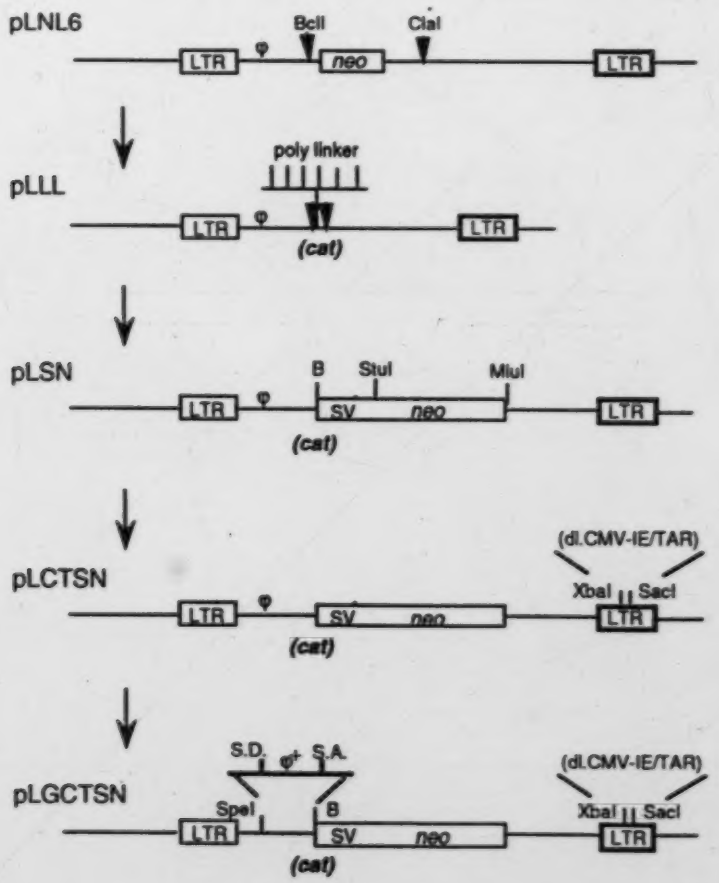


FIGURE 5. Modification of the retroviral vector. (a) Moloney MuLV 3' LTR reconstruction. The 3' LTR of pLNL6 plasmid was cloned into pSP72. Using the *Xba* I and the *Sac* I sites, we cloned PCR amplified fragment containing HIV-1 TAR or CMV/TAR to generate the pMT and pMCT plasmids, respectively. A cat reporter gene and a SV40 polyadenylation signal were cloned downstream to generate pMT-cat and pMCT-cat. (b) Reconstruction of two-LTR retroviral vectors. pLNL6 was modified by deleting the neo gene between the *Bcl* I and the *Cla* I sites and inserting a polylinker to create the pLLL vector. To generate the pLSN construct, a SVneo gene cassette was cloned into the polylinker between the *Bam*HI (B) and *Mlu* I sites. To generate pLCTSN, a fragment containing the modified LTR from pMCT was used to replace part of the 3' LTR of pLSN. The *Spe* I-*Bam*HI fragment of pLCTSN was then replaced with a fragment of pDGLtax/rex containing an extended packaging and 3'splice site (+ and S.A.) to generate the pLGCTSN. A cat gene was inserted in the polylinker region upstream of the SV-neo gene of each of the pLLL-derived vectors to generate the CAT reporter constructs.

A.


(HIV-1/TAR)
pMT-cat
XbaI
SacI
LTR
MuLV
cat
SV40 polyA
(dl.CMV-IE/TAR)
pMCT-cat
XbaI
SacI
LTR
MuLV
cat
SV40 polyA

B.


pLNL6
BclI
ClaI
LTR
φ
neo
LTR
pLLL
poly linker
LTR
φ
(cat)
LTR
pLSN
B
StuI
MluI
LTR
φ
SV
neo
(cat)
LTR
pLCTSN
(dl.CMV-IE/TAR)
XbaI
SacI
LTR
φ
SV
neo
(cat)
LTR
pLGCTSN
S.D.
φ+
S.A.
(dl.CMV-IE/TAR)
SpeI
B
XbaI
SacI
LTR
SV
neo
(cat)
LTR

of LNL6, the cat gene and a SV40 polyadenylation site. To create the plasmid pMT-cat, the CAAT box upstream of the MLV LTR TATA box was replaced with the HIV-1 TATA/TAR. pMCT-cat was generated when this site was replaced with both the dl.CMV-IE and HIV-1 TATA/TAR elements (Figure 5a). The promoter activities of the two hybrid constructs were compared with the wild-type MLV LTR, HIV-1 LTR, and the CMV-IE. This was done with and without co-transfecting a HIV-1 Tat expressing plasmid and using a series of different human cell lines; HeLa, two hepatoma cell lines (HepG2 and Huh7) and two lymphoma cell lines (CEM and H9). Results of this study are summarized in Table 2.

When Tat was included in the same system we observed that in general only pMCT-cat construct was able to respond to the transcriptional activator (Table 2 and Figure 6). This is seen consistently in all cell types examined. pMT-cat did not appear to increase its transcription rate in response to the activator (Table 2 and Figure 6). Tat poorly activates HIV-1 promoters that do not contain either SpI or NF-κB motifs (27, 28, 324), neither site is present in pMT. When these promoters are analyzed without Tat it is clear that the changes in pMCT-cat result in a promoter with a higher basal level of transcription than the parent (Table 2). It is likely that this is due solely to the inclusion of the CMV element in this promoter as the pMT-cat plasmid had a promoter strength comparable to the parent. The LTR containing the CMV element did not result in a promoter that is as strong as CMV promoter alone, in these cells.

Not surprisingly promoter activity varies with cell type. To characterize the pMT and pMCT constructs further, Dr. Chang examined the function of these promoters in a human B-lymphoblastoma cell line (AA-2). AA-2 cells are a fusion of B and T cell lines (58) and

TABLE 2. Relative levels of promoter strengths of chimeric MoMLV LTR constructs *versus* HIV LTR and CMV promoter in different human cell lines.

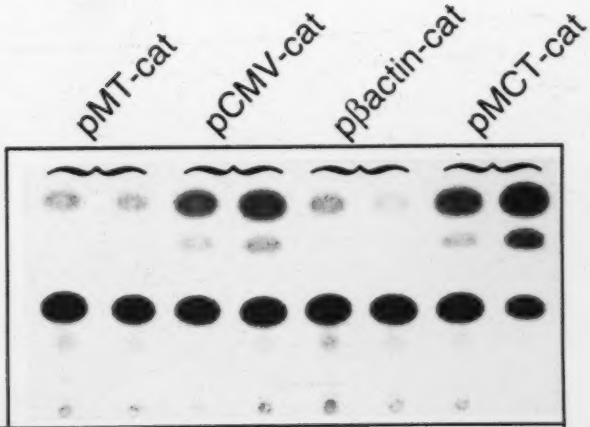
Cells	Plasmids				
	pMoLV-LTR-cat	pMT-cat	pMCT-cat	pHIV-LTRcat	pCMV-cat
HeLa	3.3/4.0*	3.5/2.2	9.0/36.3	1.0/34.7	45.2/19.9
HepG2	0.6/0.5	0.2/0.2	5.4/11.5	1.0/10.7	12.1/14.2
Huh7	0.7/1.0	0.4/0.5	7.7/19.0	1.0/22.0	18.6/ND
CEM	24.0/28.0	2.9/10.0	2.2/35.0	1.0/30.0	52.8/48.1
H9	13.0/ND	0.2/0.2	5.6/63.0	1.0/42.0	43.0/ND

*(-tat/+tat Relative levels of CAT expression)

Results are relative to the pHIV LTR-cat minus tat within each group of cells based on more than three assays. Transfection was normalized with human growth hormone gene expression for HeLa, HepG2 and Huh7 cells. ND, not determined.

FIGURE 6. Effect of Tat (transient) on B lymphoblastomas expression from the pMT and pMCT constructs. Comparison of promoter activities in AA-2. AA-2 cells were electroporated and the expression of pMT-cat and pMCT-cat was compared with two other promoter constructs, CMV-IE and human β-actin. In this case the CAT assay shown in the figure used different quantities of cell lysate in the different lanes because the pMCT-cat expressed extremely high levels of CAT, especially in the presence of Tat, and would be out of the linear range. However, the number indicates the relative levels of CAT, normalized for transfection efficiency and for total quantity of protein in each lysate. For example in the presence of Tat the pMCT-cat plasmid expressed 140 times more CAT than pMT-cat. Figure prepared by LJC.

Promoter Activities in AA2 Cells -/+ TAT



TAT :	-	+	-	+	-	+	-	+
Relative CAT Expression:	1	1	12	15	1	0.7	30	140

this cell line is extremely sensitive to HIV infection. For comparison a cellular β-actin promoter construct as well as the CMV-IE promoter were included and all promoters were again assayed in the presence or absence of Tat (Figure 6). The pMCT-cat construct had the highest level of expression with or without Tat and was shown to be Tat inducible in this system. The high levels of expression observed in this system may be due to the presence of heterologous viral transactivators in the AA-2 cells as this line was established by viral (EBV)-transformation (386). When extrapolating from AA-2 cells to expected promoter performance in corresponding untransformed cells one must consider the presence of the transactivators.

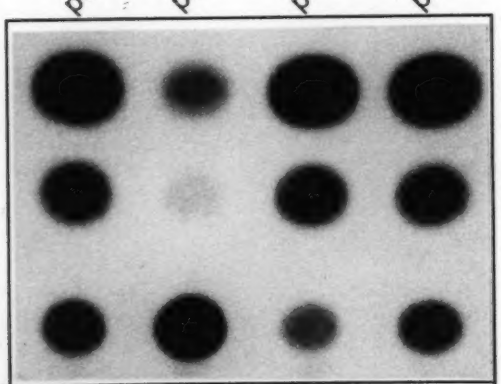
Expression levels from pMCT-cat in the presence of Tat were further studied using a human T lymphoma cell line, CEM-TART, which constitutively expresses HIV-1 Tat (64). CEM-TART cells were electroporated with four different plasmids: pCMV-cat, pLLL-cat, pMCT-cat and pHIV LTR-cat and relative levels of CAT expression were determined (Figure 7). In the presence of Tat, pMCT is again at least 30 times more active than the wild-type MLV LTR(pLLL-cat), and about two times more active than either the HIV LTR or CMV-IE constructs.

3. Levels of transcription from the two initiation sites of pMCT

The pMCT construct has TATA boxes derived from MLV and HIV-1. It is possible that transcriptional initiation could occur from either of these sites. To test whether initiation occurs from both sites and the effect, if any, that Tat may have on the initiation, RNA synthesized by pMCT-cat ± a Tat expressing plasmid (pCEP-*Tat*) was harvested and analyzed by Northern blotting and RNase mapping (Figure 8). If both initiation sites are

FIGURE 7. pMCT-cat activity in CEM-TART cells which constitutively express Tat. CEM-TART cells were electroporated with DNA as indicated and the relative CAT activity of CMV, HIV LTR and pMCT was compared with pLLL-cat, a construct that contains the wild-type MLV LTR.

Promoter Activities in CEM-TART Cells



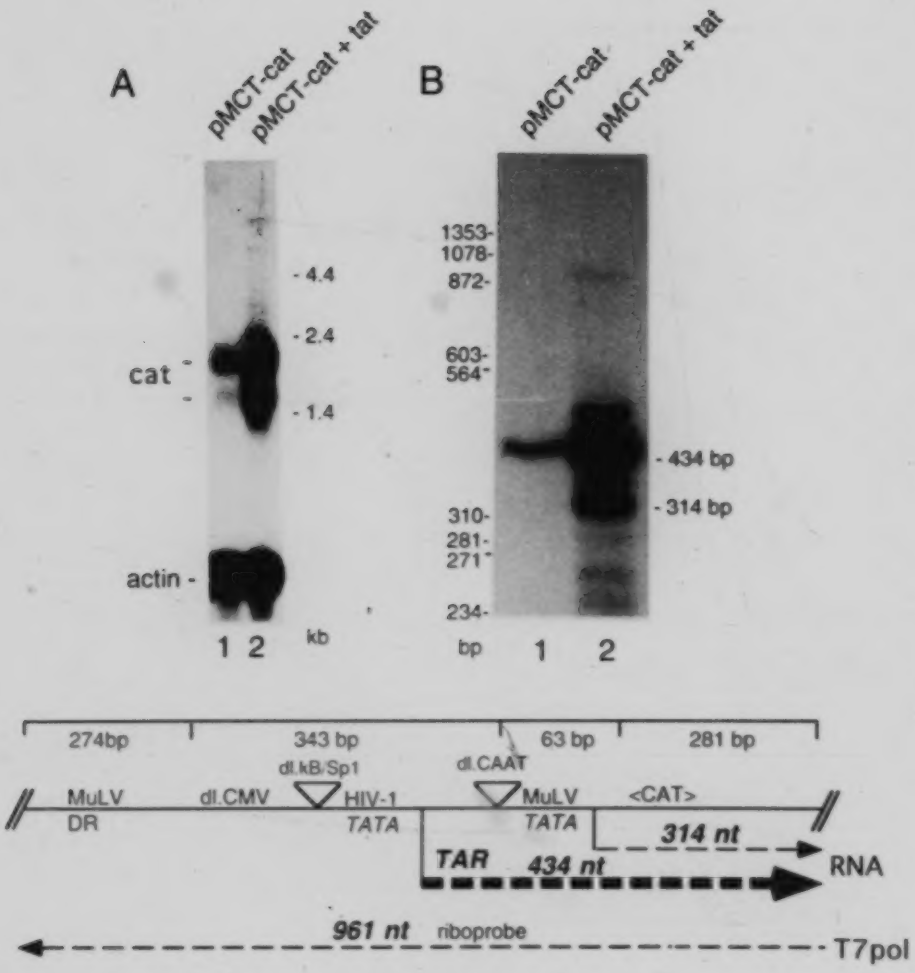
Relative CAT Expression:	18	1	30	16

used, two species of protected RNA of 434 nucleotides (nt) and 314 nt are expected. The Northern blot in Figure 8a does not provide any information about the number of initiation sites used. The agarose gel would not be able to separate the two RNA species produced if both initiation sites are used. The probe is reacting strongly with two bands in each lane of the Northern blot, the larger band is the correct size to be the full length transcript produced by this construct and we can only speculate as to the origin of the smaller species. It is possible that the smaller species is a spliced derivative of the full length transcript as MLV vector contain cryptic splice sites (183). Other possibilities include cross hybridization to other cellular RNAs. To partially characterize the initiation sites, a more detailed type of analysis was used. Transcriptional initiation sites were characterized by RNase mapping using a riboprobe as illustrated in the bottom of Figure 8. As the riboprobe was continuously labeled it is not possible to make a conclusive assessment of the precise start sites of the two transcripts. However, the data supports the notion that both initiation sites were used, and the upstream initiation site appears to be preferred eight to ten fold over the downstream MLV site (Figure 8b). Tat upregulated synthesis from both promoters 17- fold from the HIV promoter and 22-fold from the MLV promoter. This data was obtained by using a Fuji BAS1000 phosphoimager to quantitate the viral RNA signal (retroviral RNA was normalized using the actin signal). This suggests that the presence of TAR/Tat at the upstream promoter can influence transcription from the downstream location. Tat has been shown to influence transcription from TAR-less promoters and appears to be behaving like a conventional transcriptional activator (27, 69, 173, 339).

FIGURE 8. Analysis of transcription initiation from the pMCT-cat construct in HepG2 cells, in the presence or absence of Tat. RNA from HepG2 cells transfected with pMCT-cat alone (lane 1) and pMCT-cat plus pCEPtat (lane 2) was analyzed by Northern blot and RNase mapping.

(a) Northern analysis. Cytoplasmic poly A+ RNA from transfected HepG2 was analyzed on a 1.6% formaldehyde agarose gel. Two species of CAT RNA are indicated, they are approximately 2000 and 1500 bases in length. To control for the quantity of RNA loaded, the same blot was stripped by heat denaturing the CAT probe and rehybridized with a β-actin probe. RNA molecular weight markers are shown on the right.

(b) RNase mapping. Cytoplasmic polyA+ transcribed from the pMCT-cat construct was hybridized to the riboprobe shown at the bottom of the figure and then treated with RNases. The two major protected RNA species that arise are consistent with the two different transcription initiation sites present in pMCT. Figure prepared for LJC.


A
pMCT-cat
pMCT-cat + tat
- 4.4
- 2.4
cat
- 1.4
actin -
1 2
kb
B
pMCT-cat
pMCT-cat + tat
1353-
1078-
872-
603-
564-
- 434 bp
- 314 bp
310-
281-
271-
234-
bp
1 2
274bp
343 bp
63 bp
281 bp
dl.kB/Sp1
dl.CAAT
MuLV
DR
dl.CMV
HIV-1
TATA
MuLV
TATA
<CAT>
314 nt
TAR
434 nt
RNA
961 nt
riboprobe
T7pol

4. Generation of retroviral vectors with modified LTRs

pMT and pMCT are single LTR plasmids, whereas true retroviral vectors have two complete LTRs. To create a new gene therapy vector Dr. Chang started with the existing vector pLNL. To facilitate future cloning, the neo resistence marker of pLNL was replaced with a polylinker. With pLLL as the basis, a series of retroviral vectors were created: pLSN, pLCTSN and pLGCTSN all contain a SV40 promoter-*neo* selective gene. Versions of each of these were created that contained the *cat* reporter gene. pLSN is the wild type LTR vector, pLCTSN contains a modified 3' LTR derived from pMCT and pLGCTSN contains the modified LTR as well as an extended packaging signal and a 3' splice acceptor derived from a previously constructed vector (6). For details on the construction of the extended packaging signal see Figure 12. The extended packaging signal was added to improve packaging efficiency and to avoid problems associated with cryptic splicing due to the presence of a 5' splice donor. It was felt that the 5' splice donor may use a cryptic splice acceptor present on a therapeutic gene. Enhancer/promoter modifications were made to the 3' LTR since these will appear in both LTR's following transcription, reverse transcription and integration. The therapeutic gene is added to these vectors between the packaging signal and the selectable marker (Figure 5).

5. Relative virus titer of modified retroviral vectors

To examine if the extended packaging signal in pLGCTSN would result in more efficient encapsidation of vector sequences in viral particles, the packaging efficiencies of pLNL6, pLSN, pLCTSN and pLGCTSN were studied in the PA317 packaging cell line. Duplicate plates of PA317 cells were transiently transfected with equimolar amounts of one

of the retroviral vector DNAs using lipofectamine, and supernatants were harvested after 48 h. HeLa and Huh7 cells were transduced with the recombinant vectors and viral titer determined by counting G418 resistant colonies. Viral titers of pLCTSN and pLGCTSN vectors were 3 to 10 times higher than the parental pLNL6 or pLSN vectors (Table 3). This result demonstrates that the modified LTR's do not interfere with packaging, reverse transcription and integration. The higher titers of pLCTSN and pLGCTSN may reflect an increase in the efficiency of packaging or higher yields of genomic RNA or higher efficiency of expression of Neo from the integrated provirus. It is interesting that pLGCTSN's extended packaging signal did not improve viral titer above that of pLCTSN. Perhaps the splicing is so efficient the relative levels of the spliced vs the unspliced is very high.

6. Levels of expression after long-term integration

Long term expression from the stably integrated LTR was examined in HepG2 and HeLa cells. Virus produced from transfected PA317 was used to transduce HepG2 and HeLa cells. Transduced cells were harvested 1-2 months after selection with G418. An equivalent number were lysed and CAT activity per unit protein was determined. In both cases, the vector containing the modified LTR (pLCTSN-cat) had higher levels of CAT expression than pLSN-cat with a greater difference apparent in the HepG2 cells. To examine the effect of Tat on promoter strength a Tat plasmid was transfected into the stable HepG2 cell lines. A higher rate of CAT expression is observed in these cells, it is possible then that Tat modestly increased the transcription rate from these cells (Figure 9). These results demonstrate that expression from pLCTSN is present at 1-2 months after selection and continues to be responsive to the transactivator.

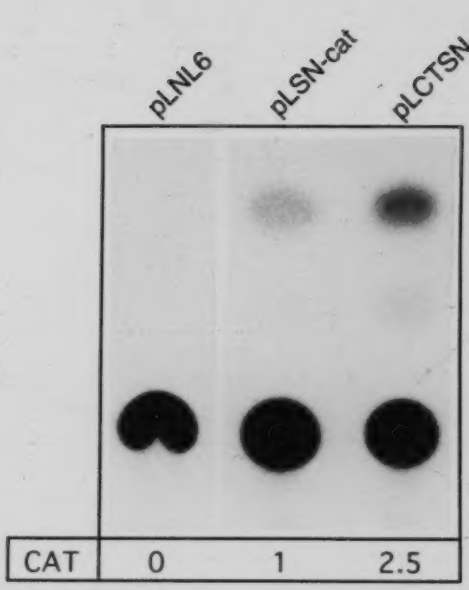
TABLE 3. Titration of packaged retrovirus by G418 selection

Cells **Vectors**

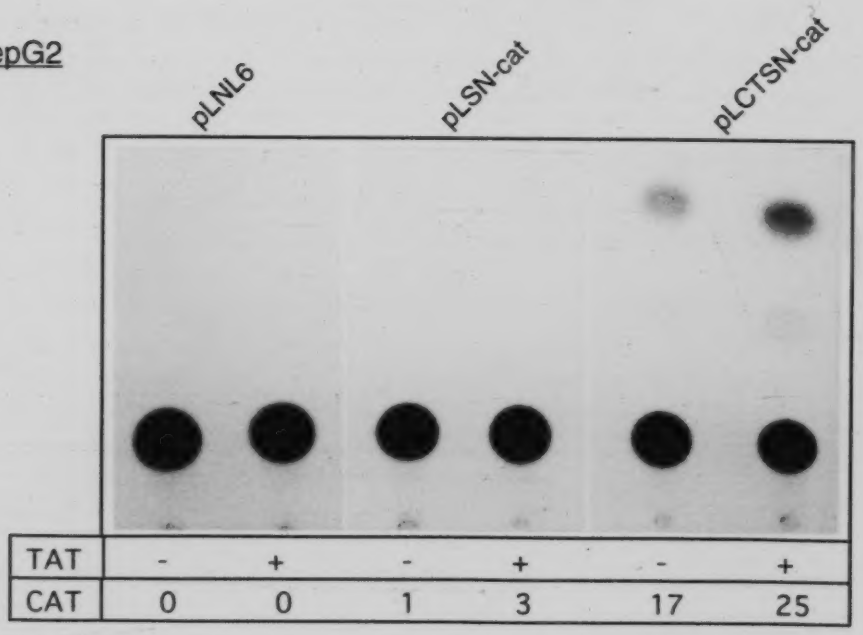
	pLNL6	pLSN	pLCTSN	pLGCTSN
HeLa titer ($x10^6$)	6.5,0.3	3.8,9.0	22.8, 15.3	35.5,21.0
Huh7 titer ($x10^6$)	2.5, 0.9, 1.0, 3.6	5.3, 0.8, 7.3, 9.7	9.0, 5.8, 7.4,9.7	12.2, 13.8, 12.7, 12.5

FIGURE 9. Long-term study of expression from various recombinant vectors. Virus was prepared from PA317 cells transfected with equal amounts of pLNL-6, pLSN-cat or pLCSTN-cat and used to infect HeLa cells. These were then selected with G418 to establish stably transduced cell lines, which were grown in culture for 1-2 months. Cells ($5X10^6$) were harvested, lysed and protein concentration determined. Lysates containing the same amount of protein were then used for the CAT assay. (a) HeLa cells. (b) HepG2 cells. HepG2 cell lines were transfected with either a Tat plasmid or with a control plasmid and CAT activity was determined 48 h later. Relative levels of CAT expression are shown. Note, the control transfections with a β-galactosidase reporter construct suggest that less than 10% of the transduced HepG2 cells would be transfected with the Tat plasmid, and therefore transactivation would be expected to be 10-fold higher if all cells had been transfected with the Tat plasmid (see Figure 10).

HeLa


pLNL6
pLSN-cat
pLCTSN-cat
CAT
0
1
2.5

HepG2

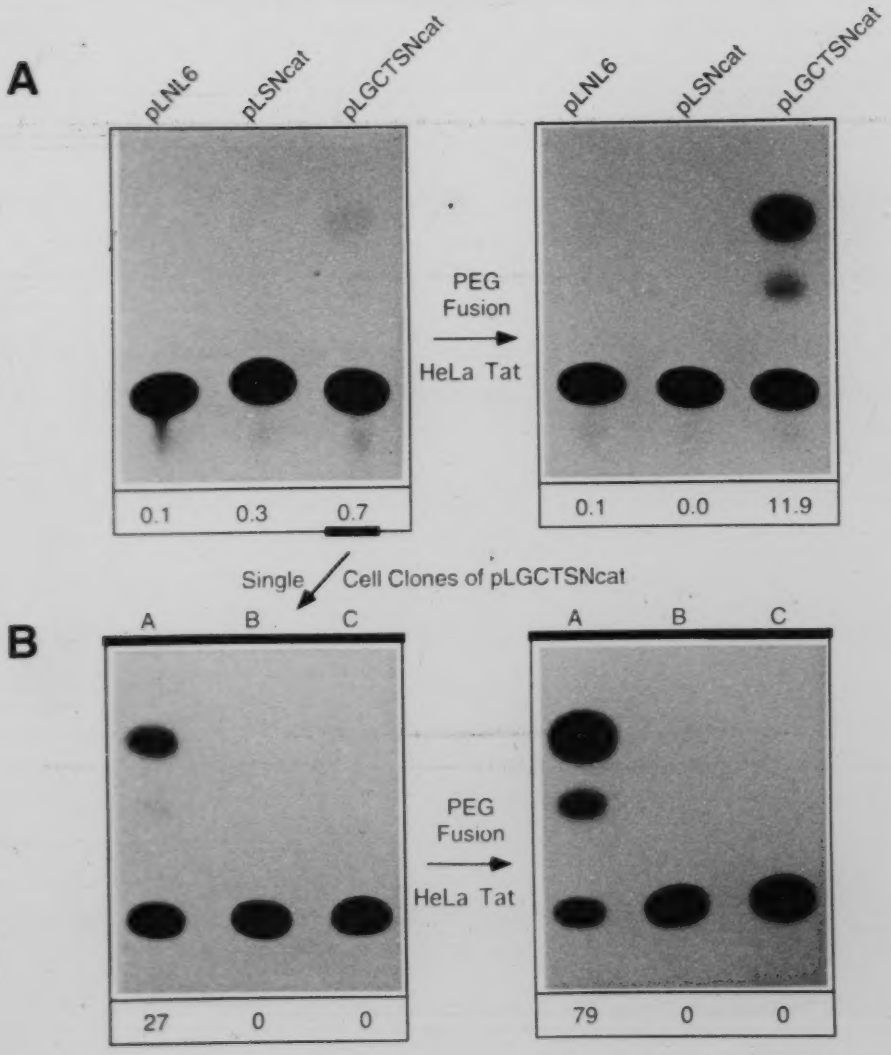

pLNL6
pLSN-cat
pLCTSN-cat
TAT
-
+
-
+
-
+
CAT
0
0
1
3
17
25

7. Transactivation of the stably integrated MLV/CMV/HIV TAR LTR by PEG fusion with Tat-producing cells

In the previous study only a low level of transactivation was observed in the cell lines transfected with a Tat producing plasmid, possibly due to the inefficiency of transfection. To overcome this potential limitation we used a Tat producing cell line to introduce the transactivator to stable cell lines containing the vectors. Transduced and G418 selected cells expressing pLNL6, pLSN*cat* or pLCSTN*cat* were next fused by polyethlyene glycol (PEG) to a HeLa cell line, which expresses Tat constitutively. Fusion was verified by light microscopy. This cell line was produced in Dr. Chang lab and was known as HeLa-Tat. LTR-driven reporter expression was determined before and after fusion with the Tat cell line (Figure 10). pLSNcat showed little CAT activity before or after cell fusion. In contrast, pLGCTSN-cat had a two fold higher basal level of activity and a 15- to 20- fold increase in CAT activity after the introduction of Tat. As integration of a retroviral provirus is a random event, individually isolated clones were examined for LTR function. Of the three clones examined for CAT activity, only one (clone A) exhibited a high level of CAT expression, that was further activated after fusion. This study demonstrated that following long term integration the modified LTR (pLGCSTN-*cat*) is more active than the original MLV LTR (pLSN-cat) lines. A higher rate level of CAT expression is observed in these cells, it is possible then that Tat modestly increased the transcription rate from these cells (Figure 9). These results demonstrate that expression from pLCTSN is present at 1-2 months after selection and continues to be responsive to the transactivator.

FIGURE 10. Transactivation of integrated reporter gene by fusion to HeLa Tat cells. (a) CAT assay of transduced HeLa cells before and after PEG fusion to a line of HeLa cells that express Tat. HeLa transduced with pLNL6, pLSN-cat or pLGCTSN-cat and selected in G418 for three weeks were plated together with the HeLa Tat cells 24 h before PEG fusion. CAT assay was performed for 2 h using lysates containing the same amount of protein (200 mg). (b) CAT assay before and after PEG fusion of individual cell clones isolated from the pLGCTSN-cat transduced HeLa cells. CAT assay was performed using lysates containing the same amount of protein (50 mg).

HELA TAT FUSION TRANSACTIVATION



D. Discussion

1. Gene therapy vector LTR modifications

This thesis is aimed at creating a retroviral vector that would improve an existing MLV-based vector in expression and titer. One goal of this study was to alter the MLV LTR so that it would more efficiently drive gene expression via Tat in cells of the hematopoetic lineage. Pluripotent stem cells are the target tissue for many gene therapy applications, including anti-HIV therapy. This tissue is capable of self-renewal and many diseases treated by gene therapy require that the corrective protein secreted by the treated cells enter systemic circulation. Furthermore, because HIV predominantly infects cells of the hematopoetic system, pluri or multipotent stem cells are potential targets for introduction of antiviral genes. This might result in the presence of the gene in functional hematopoetic cells following proliferation and differentiation of the stem cells.

In more detail, the LTR of the originating vector, pLNL6, was altered so that it contained a version of the CMV-IE enhancer and the HIV derived TAR (62). The CMV immediate early promoter is functional in both lymphocytes and monocytes (284). A 228-bp CMV enhancer element, derived from the full length promoter, was placed upstream of the MLV transcriptional start. The CMV enhancer is a strong transcriptional activator with ubiquitous gene expression properties (42). This 228-bp element contains binding sites for κB regulatory elements and an Ap-1 binding site (136, 199). These sites are recognized respectively by NF-κB and Fos-Jun, both of which are stimulated transiently following T-cell activation. It was felt that promoters up-regulated with differentiation could also upregulate

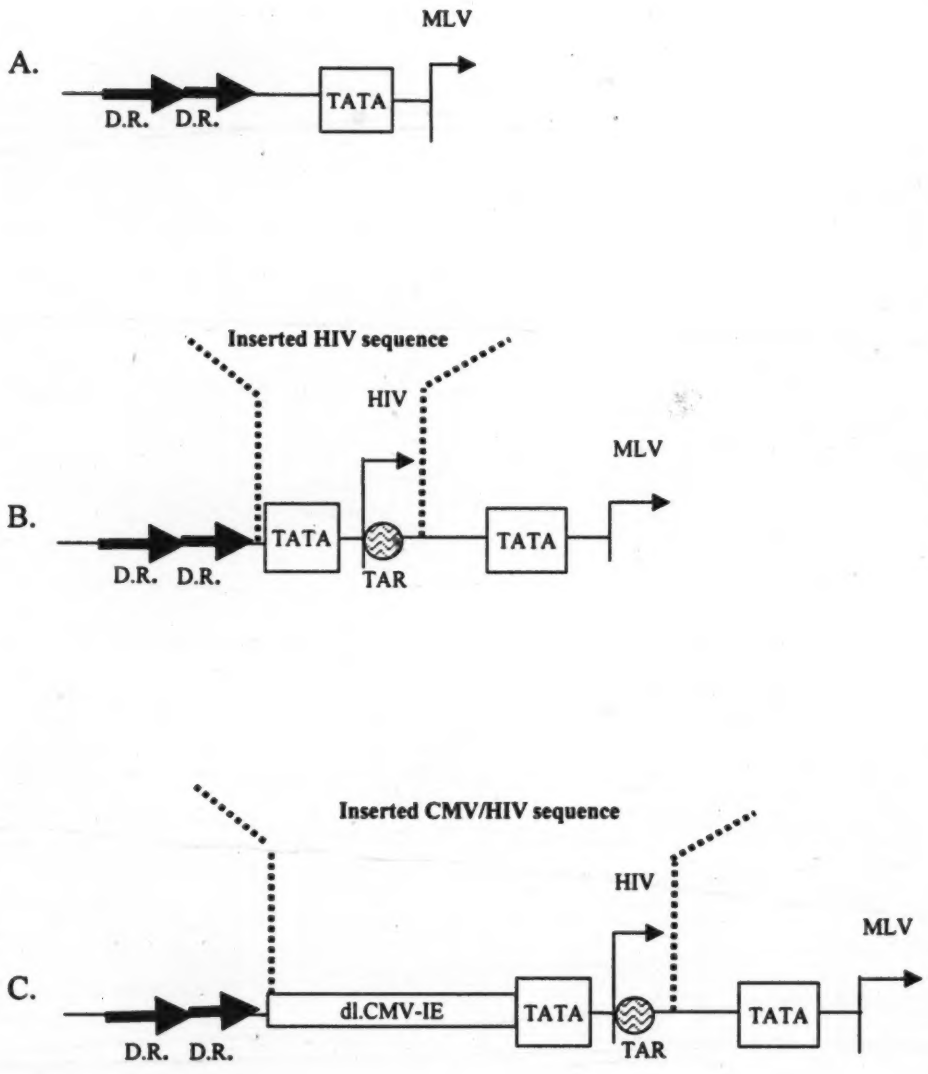
expression of an exogenous gene in a retroviral vector. This would allow expression of the gene to be 'turned-up' when these cells differentiated.

Although it was hoped that a vector containing this LTR would be useful overall for gene therapy applications its' specific purpose was to direct the expression of anti-HIV genes. Gene therapy for HIV is dependent on the maximization of therapeutic gene expression in CD4+ lymphocytes. Thus, therapeutic moieties should be up-regulated because of HIV infection giving optimal protection during high turnover of the virus. The TAR element of HIV was included in the promoter design as it was felt that TAR would provide the HIV inducible function. TAR is the target sequence for the HIV regulatory protein Tat. Tat is necessary for efficient expression from the HIV LTR. Without Tat, transcription initiating from the viral LTR terminates prematurely within several hundred nucleotides and only short RNA's are recovered (174). Tat is an RNA binding protein. The Tat target sequence, TAR, is present in the newly transcribed R region of the 5' LTR. In HIV, Tat function has been uniformly invoked to interact in a sequence-specific manner with the cis-acting viral element TAR. TAR can form a stable stem loop structure (244). Mutations that change the sequence in the stem loop or disrupt the stem structure, greatly reduce the transactivation by Tat (118, 309). Tat is normally found only in cells infected with HIV. A vector containing the TAR element could provide a means by which foreign genes that interfere with HIV could be up-regulated in response to HIV infection.

Initially the proposed modifications to the LTR were analyzed using expression plasmids that consisted of an altered LTR driving the expression of the reporter gene *cat*. Two expression plasmids were created: pMT and pMCT. pMT-cat used the MLV LTR as

a backbone. It incorporated the HIV TATA and TAR elements. pMCT-cat, also based on MLV, included HIV TATA and TAR as well as the deleted version of the immediate early promoter of CMV (IE-CMV) (Figure 11). These two constructs were designed to allow for the identification of a synergistic effect between TAR and the CMV-derived sequences. Comparison of pMT-cat with pMCT-cat revealed that the former did not express efficiently at the basal level nor was it significantly induced by Tat (Table 2 and Figures 7 and 8). This was the case in all the cell lines examined except CEM where some degree (five-fold) of Tat induction could be observed for p-MT (Table 2). pMCT-cat in the presence of Tat showed expression levels comparable with a pHIV-LTR-cat construct, indicating that this promoter can produce transcripts at levels comparable with HIV in infected cells. The pMT-cat results were initially surprising, as it had been expected this construct would be induced in the presence of Tat. These results are explained by the effects that upstream HIV and heterologous promoter elements had on the levels of basal expression, and the function of TAR and Tat. The complete HIV promoter contains three SpI binding sites (294) and two NF-κB binding sites (249) which are functionally important in basal and trans-activated expression. Tat poorly activates HIV-1 promoters which do not contain either SpI or NF-κB motifs (27, 28, 324). It is important to note that neither site is present in pMT. Berkhout and Jeang created a reporter system that had only a minimal piece of the HIV promoter. This system included TAR and 43 bases upstream from the start of transcription but did not contain the SpI and NF-κB binding motifs this promoter was not responsive to Tat. Addition of short synthetic enhancer motifs (ApI, Oct, SpI and NF-κB) could confer Tat responsiveness to the minimal HIV-LTR (29). Two roles can be considered for enhancers

FIGURE 11. Details of the promoter elements of MLV LTR, pMT LTR and pMCT LTR. A. The MLV LTR, D.R. direct repeats. B. pMT, shown with the inserted HIV TATA and TAR elements, note that the MLV start is retained. C. pMCT shown with the inserted chimeric CMV/HIV sequences.


A.
MLV
D.R.
D.R.
TATA
B.
Inserted HIV sequence
HIV
MLV
D.R.
D.R.
TATA
TAR
TATA
C.
Inserted CMV/HIV sequence
HIV
MLV
D.R.
D.R.
dl.CMV-IE
TATA
TAR
TATA

in Tat *trans*-activation. First, enhancers may prime the promoter to provide a basal level of transcription to make TAR RNA available for Tat attachment. Second, enhancer binding proteins could be a part of a multiprotein complex that includes cellular factors and Tat. Correct formation of this complex could lead to enhanced transcription. Huang show that spacing of the upstream promoter elements from TATAA and TAR is critical for proper transactivation by Tat, thereby supporting the second model (161).

The promoter contained in the pMT-cat construct is fully derived from MLV. This promoter contains two direct repeats for which no corresponding transcription factors have been identified. In this context (pMT-cat) the MLV promoter is incapable of up-regulating the TAR/Tat complex. pMCT-cat places CMV enhancer elements proximal to the HIV TATAA and it is this juxtaposition allows for the transactivation of this initiator by Tat.

The cloning design of pMCT retains the original MLV TATAA box, located downstream of the inserted HIV TATA and TAR elements. In examining expression from pMCT-cat, another interesting effect is that RNase mapping implies both initiation sites are used in this construct and both are upregulated by the presence of Tat. Trans-activation of the MLV promoter by Tat is a particularly interesting result as the MLV transcript does not have a transcribed TAR (Figure 11). This result supports our previous observation in pMT-cat; that elements upstream of MLV promoter's start site were regulating Tat activation of this system. The activation of both promoters in pMCT-cat confirms other work which shows that Tat can exert its effect on transcription levels by being near, but not necessarily attached to, a nascent RNA transcript. In a study using a GAL4-Tat fusion protein, a promoter containing GAL4 binding sites was transactivated without TAR (173). This study

proved that Tat could function like a typical transactivator by activating a promoter through binding to the DNA upstream from its transcriptional start. Other groups have targeted Tat to DNA using a Tat-Jun construct (27). In support of the notion that Tat may function as a general transcription factor and its interaction with TAR is merely to tether it to the location of the elongating polymerase, studies have shown that Tat can transactivate heterologous promoter elements that are TAR-less (184). Tat has been shown to transactivate the JC (Jamestown Canyon) virus late promoter (339) and augments gene expression and viral replication of human CMV (157). Tat has been shown to enhance the efficiency of transcriptional elongation (88, 126, 134, 169).

We know from the previously discussed experiments that Tat, if tethered upstream on DNA, can transactivate heterologous promoters. Also, we know that TAR/Tat, if included on a heterologous transcript, will transactivate this system. However none of these studies examines the effect of a TAR/Tat interaction on surrounding promoters. A recent paper examined the interaction of a promoter containing TAR with a second promoter (374). This system used part of the c-myc gene including its two promoters, P1 and P2, and a site of premature transcription arrest. In this study the TAR element was placed immediately downstream from the P2 promoter such that TAR would be included in transcripts originating from P1 and P2. It was shown that Tat could activate transcription from the P2 promoter when TAR was as far away as 353 nucleotides downstream, and surprisingly from the P1 promoter when TAR was 518 bases downstream. This result conflicts with an earlier report that efficient activation of the HIV LTR by Tat required precise positioning of TAR immediately 3' to the site of transcriptional initiation (309). It however may be the case that

low efficiency of HIV transcriptional initiation may account for this difference and the c-myc promoter, which displays more efficient initiation, would not exhibit the positional constraint.

Several different models for the effect of TAR on the elongation of the RNA polymerase complex have been proposed. The work showing that TAR must be positioned next to the start site was interpreted to imply that the Tat/TAR complex could loop back and interact with a newly initiating RNA polymerase at the promoter (309). The fusion protein systems that converted Tat interaction from RNA to DNA allow for the same looping back mechanism.

Wright and Luccarini argue against the looping back mechanism due to the distances involved in their activation studies (374). They argue that 500 bases are too far away for the looping back to be mechanistically possible. Instead, they promote the idea that Tat is introduced into the transcriptional complex during its transit through TAR, thus resulting in the modification of the transcriptional complex to an elongation competent form. Their model implies that Tat would most likely affect only the transcriptional complexes on the nascent transcript where it is attached via TAR.

Although we have not conclusively shown that both promoters are active in pMCT, the result from the RNase protection study implies that this is the case. If the reader is willing to accept that both promoters are functional in pMCT then the data presented here argue against the necessity of Tat/TAR being able to interact only with polymerases that are on the transcript containing this complex. This work implies that the Tat-TAR complex can

interact with a downstream promoter that does not contain the TAR element either as DNA or RNA.

The hybrid promoter contained in pMCT-cat has two transcriptional start sites 120 bases apart. The TAR element is only contained in the transcript produced by the upstream promoter, yet RNA produced by both promoters is increased by the presence of Tat. Our work more closely agrees with the theory where the Tat/TAR complex can loop back or ahead to influence an elongating polymerase as the most probable means for Tat to influence the TAR-less promoter would be through the looping mechanism. In this way the Tat/TAR complex can reach the polymerase on the TAR-less transcript. In any case, the function of Tat is complex. The "real" mode of Tat transactivation may show that Tat/TAR also behaves as an enhancer element and can influence transcription over great distances up and downstream of its location.

Our system might be useful in determining from what distance Tat/TAR could be transactivate promoter elements. This result has some implications for Tat/TAR transactivation of cellular promoters near integrated proviral HIV. It would also be interesting to examine the distances at which Tat/TAR can activate transcription and whether it would activate normal cellular promoters. This may not contribute to HIV pathogenicity in any major way, however it may have implications as to cellular effects of gene therapy vectors based on HIV or those that included only the TAR element.

2. Generation of high titer recombinant vectors

One serious drawback for clinical gene therapy applications has been the difficulty in readily producing high titer recombinant retrovirus. Many applications require that the

recombinant virus titer must be at least 10^6 infectious units/ml. For infectious retrovirus produced within 48 h following transfection of packaging cell lines, virus titer is generally low (10^3-10^4 /ml) (238). One way of overcoming this is to select single cell clones that produce retrovirus at a higher titer. This is a long process that can take 1-2 months and must be repeated for each different construct. During the prolonged selection process, gene(s) encoded by the retroviral vector may inhibit growth of the packaging cell line, favouring the outgrowth of clones that express low levels of virus (269).

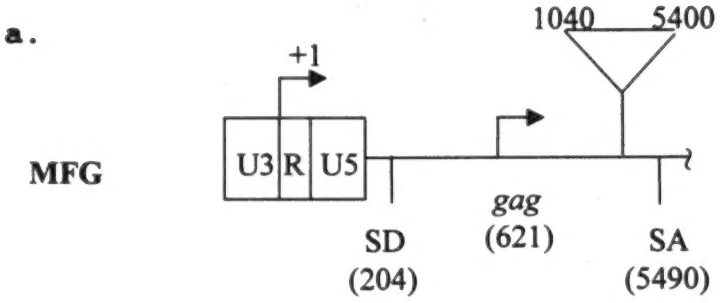
This study explored a means of producing viruses by optimizing transient transfection which has the benefit of reducing the time and effort required to produce high titer retroviral stocks. The approach taken in this thesis was to engineer a vector with an improved packaging signal. A vector with more efficient packaging can produce viruses in higher titers. The vector pLCTSN was modified to contain an extended packaging signal altered to encompass a splice donor and splice acceptor region. This modified vector was called pLGCTSN.

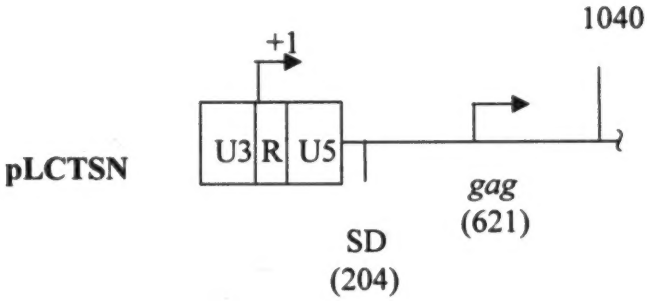
Figure 12 is a drawing of part of the retroviral vectors; pLNL6, MFG and pLGCTSN. The drawing shows the relative lengths of the packaging signal sequences present on each vector. We chose to include ~1500 bases on the gag coding region in pLGCTSN as it had been demonstrated that constructs containing sequences that extended into the gag coding region of MLV produce more viruses (1, 6, 13, 24). (The MLV sequence numbers are as previously described (351).

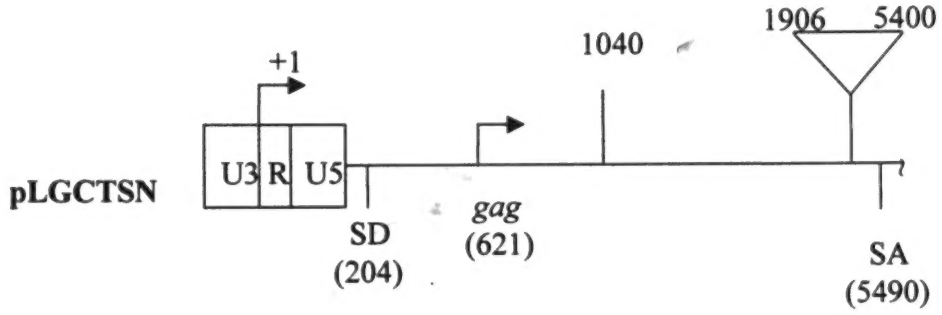
To test the extended packaging signal, pLGCTSN was transfected into the packaging cell line PA317 to produce viruses for titering. For comparison, pLNL6, pLSN and pLCTSN

FIGURE 12. Diagram of the packaging signal in MFG, pLCSTN and pLGCTSN. The numbering system is based on VanBeveren,et al (351). MFG contains gag sequences up to the *Nar* I site at position 1040 followed by a splice acceptor fragment from the *Nde* I site (position 5402) to the *Xba* I site (position 5766) in MLV. LCTSN contains gag sequences up to position 1040. LGCTSN includes a portion of the gag gene up to the *Bgl* II site (position 1906), the env splice acceptor is included on a fragment from *Bgl* II (position 5407) to *Sma* I (position 5776). b) Partial restriction map of MLV.

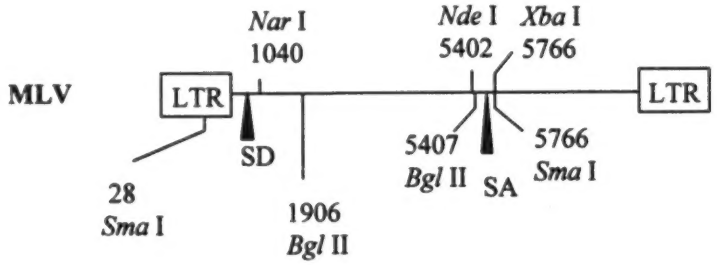
a.


1040
5400
+1
MFG
U3
R
U5
gag
(621)
SD
(204)
SA
(5490)


1040
+1
pLCTSN
U3
R
U5
gag
(621)
SD
(204)


1906
5400
1040
+1
pLGCTSN
U3
R
U5
SD
(204)
gag
(621)
SA
(5490)

b.


Nar I
1040
Nde I
5402
Xba I
5766
MLV
LTR
LTR
SD
28
Sma I
1906
Bgl II
5407
Bgl II
SA
5766
Sma I

were also transfected into PA317. The viruses harvested from the transfected cells were used to transduce HeLa or HepG2 cells. Successfully transduced cells were selected for the neomycin resistant phenotype using G418. Finally titer was determined by counting the G418 resistant colonies. The results of this experiment are shown in Table 3.

Unexpectedly, the viruses produced by the pLCTSN and pLGCTSN constructs titer at approximately ten fold higher levels than pLNL6 and pLSN. This is unexpected because only pLGCTSN construct had the extended packaging signal. This result suggests that the extended packaging signal did not increase the titer. Although it had been reported that longer sections of gag increased viral titer at the time this study was undertaken, the region reported to be responsible for packaging had not been subjected to a detailed mutational analysis. After this thesis work Kim et al analyzed the packaging region of the retroviral vector MFG (183). MFG is a retroviral vector similar to pLSN and pLGCTSN in that it is based on the Moloney murine leukemia virus. Portions of the packaging region of MFG were deleted and the effect on titer determined. MFG's has the same length of packaging signal as pLCTSN, but it incorporates a functional splice acceptor as does pLGCTSN. The following results were obtained: First, mutant constructs containing deletions from position 228 to 371 completely lost the packaging function. Second, mutations from 377 to 527 seemed necessary but not essential for optimal packaging efficiency. Third, the mutant constructs which always gave the highest titer removed the entire gag coding sequence present in MFG (deleted from 523 to 1016). These results conflict with previous reports that the gag sequence contained an extended packaging signal (13, 24). Kim et al. explained these results by suggesting that the SA site may play a role in the levels of packagable RNA.

In comparison earlier work used a construct that did not incorporate a functional splicing design (13, 24). Our results do not agree the conclusion reached by Kim et al. as LCTSN does not incorporate the SA present upstream from the *env* coding region, yet its titer was as high as LGCTSN's.

The question remains why the titer of LCTSN was so high. It has the same packaging signal and uses the same promoters to express the vector genome (MLV LTR) and selectable marker (SV40 promoter) as LSN and LNL6. Our assay for packaging requires gene expression following selection of transductants. It is possible that the changes made in LCTSN compared with LSN and LNL6 could be affecting processes independent of packaging, such as reverse transcription, RNA stability or expression of the *neo* gene. About the last point, it may be true that the LCTSN constructs have higher levels of neo expression due to the proximity of the CMV enhancer sequences. Therefore the higher titers observed for both LCTSN and LGCTSN are not due to more viruses but rather, to more transductants being successfully selected. To resolve this point the selectable marker could be changed to β-gal. Successful transduction with a β-gal expressing virus could then be observed without selection.

In summary it seems that the sequences involved in packaging are more complex than originally thought and a detailed analysis of this process is necessary to isolate important regions.

3. Overview of retroviral vector design changes

A retroviral vector, pLGCTSN, was produced that had, in some cell lines, a higher basal level of expression than the originating MLV construct. A cloned heterologous gene

(e.g. *cat*) was up-regulated in response to the trans-activator, Tat. pLGCTSN has a higher titer than the originating vector. It does not have greatly increased stability and once integrated, its promoter is generally shut-down after a few months of continuous culture. When individual clones of transduced cells were grown continuously in culture and then examined for gene expression it was shown that there was a great deal of variability between the various clones (Figure 9). This result could be interpreted to mean that the variability was due to different patterns of promoter methylation between the clones. Accordingly modifications to the vector were made to try to limit the effects of methylation as described in Appendix A.

CHAPTER 3 - ANTI-HIV ELEMENTS

A. Introduction

Chapter 2 describes the development if a modified retroviral vector which expresses reporter genes at higher levels and for a longer time than the wild-type MLV vector. This chapter describes our efforts to identify an effective anti-HIV gene that could be used with the modified vector. Several different anti-HIV constructs were tested, including antisense and packaging decoy elements. Note for these studies we used the vector pLGCTSN, but for the sake of brevity we renamed it pTAR.

Antisense sequences that were complementary to portions of the HIV primer binding site, pol, tat and nef regions were tested. HIV target sequences were chosen based on their relative sequence conservation and the corresponding antisense elements were cloned into the retroviral vector pLSN. Early studies involved HeLa cell co-transfection of one of the antisense containing vectors together with a plasmid that expresses HIV-1 (pNL4-3). After transfection the cell supernatants were analyzed for reverse transcriptase (RT) activity which is associated with nascent HIV particles.

Based on the HIV-1 packaging signal, two packaging decoy elements (LJC140 and PAK140) were also constructed and tested. As is explained in more detail below, LJC140 is a fully sense version of the HIV-1 packaging signal, whereas PAK140 has a portion of the sense sequence found in LJC140 combined with 40 bases antisense to gag. PAK140 and LJC140 were cloned into the vectors pLSN and pTAR, giving rise to viruses LSN-PAK140, TAR-PAK140 and TAR-LJC140. The recombinant viruses were used to transduce two HIV sensitive human lymphoid cell lines. Stable cell lines established from these transductants

were then challenged with HIV-1 or HIV-2 viruses, and cultures followed for virus production. Recombinant retroviral RNA expression in the (uninfected) transduced cell lines was assessed by Northern blot. To examine the effect of TAR-PAK140 and LSN-PAK140 on cells that were already infected with HIV-1, these recombinant retroviruses were also transduced into chronically infected cell lines.

Finally, retroviral vectors that contained combinations of antiviral elements were examined. pTAR-PAK140 and pTAR-LJC140 were modified to contain additional gag antisense elements, and the resulting viruses were transduced in to H9 cells. The transduced cell lines were infected with HIV and followed for RT activity in the supernatant.

B. Methods

1. Construction of HIV antisense expressing plasmids

Four clones that expressed antisense transcripts to various regions of the HIV genome were constructed. Three (pLSN-antipbs, pLSN-antitat and pLSN-antinef) were constructed by PCR amplification of a portion of HIV-1. The fourth clone pLSN-antipol was made by digesting the HIV-1 proviral plasmid pNL4-3 and cloning the HIV-1 sequence into pLSN. The corresponding regions of the HIV genome that are targeted by these antisense clones are shown in Figure 22a. Details of cloning of the PCR fragments follows.

To obtain antisense fragments by PCR, a reaction was carried out using 0.01μg/μl of the PCR primers. The sequence of the oligonucleotides used as primers for amplification are given in Table 4. The enzyme used for the reactions was *Pfu* polymerase (Stratagene) and each reaction included 0.2 mM dNTP's. pNL4-3 was used as the template, at a final concentration of 0.2μg. After amplification, the products were added to 400 μl of water and

spun into (not through) a 100 micron filter at 5000 rpm for 10 min in an eppendorf microfuge. After 10 min, another 400 µl of water was added to the filter and the spin was repeated, to wash the column, 20 µl of water was added to the top, the column was vortexed vigorously, inverted into a microfuge tube and spun at 14,000 rpm for one minute to recover the PCR product.

To finish the ends, enhance the cloning success rate of the PCR products and to add phosphate groups to the 5' ends, 20 µl of the purified PCR products were processed using T4 DNA polymerase (NEB) and polynucleotide kinase (Promega). The reaction was done in T4 DNA polymerase buffer (50mM NaCl, 10mM Tris-HCL, 10mM $MgCl_2$, 1 mM dithiothreitol) (pH 7.9). Each reaction was supplemented with 0.02 µg of dNTP's and incubated at room temperature for 20 min. To stop the reaction, 1 µl of 0.5 M EDTA was added to the 25 µl reaction mixture. The resulting blunt ended and phosphorylated PCR products were run on a 1% agarose gel and bands excised. Contaminating agarose was removed by 'Gene Cleaning' (Bio 101). The PCR fragments were ligated into the *Eco*RV site of pbs-ks (Stratagene). The various PCR fragments were digested from pKS using *Bam*HI and *Hind*III and cloned into pLSN. pLSN-antipbs, pLSN-antitat and pLSN-antinef were made using this strategy, pLSN-antipol was made in the following manner. pLSN-antipol was made by *Hinc*II and *Eco*RV digestion of the plasmid pNL4-3. The digestion released a blunt ended 458 bp fragment of HIV-1 (2520-2981 bp) that was subsequently ligated into the *Eco*RV site of pBS-ks-(Stratagene) to generate the clone pKS- anti-pol. This fragment was then digested from pKS using *Bam*HI- *Hind*III and cloned into pLSN. All

TABLE 4. Oligonucleotides for PCR amplification of HIV-1 antisense constructs.

Clone	Oligonucleotide sequence	Target in HIV-1 (base #)
pLSN-anti-pbs	1.GTAGCTAGCGGTCTCTCTGGTTAGACCAG 2.GTAGCTAGCAAGTCCCTGTTCGGGCGCCC	455-653
pLSN-anti-Tat	1. GTAGCTAGCTGTTTTTCAGAATCTGCTAT 2. GTAGCTAGCACTGGCTCCATTTCTTGCTC	5381-5831
pLSN-anti-nef	1. GTAGCTAGCCGTTTCAGACCCACCTCC 2. GTAGCTAGCTTGCCACCCATCTTATAGCA	8361-8777

*HIV-1 numbering according to Myers et al. The sequence corresponding to the restriction enzyme site *Nhe*I is underlined.

clones made for this study were verified by restriction mapping, except for pLSN-antipbs which was verified by sequencing.

2. Construction of the LJC140 and PAK140 retroviral vectors.

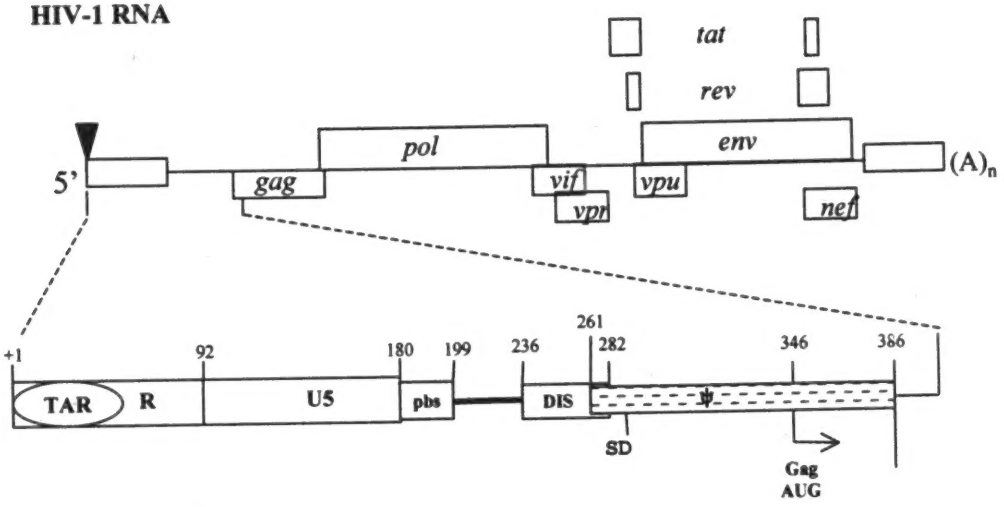
Both LJC140 and PAK140 were based on the HIV-1 packaging signal, details of which are shown in Figure 13. The sense portion was not made exactly to consensus for HIV-1, but included mutations to cripple the splice donor and gag AUG codons (Figure 13b). As we wanted to create this region with the precise mutations as outlined and include some restriction sites, a series of overlapping oligos was used in the construction of this region, their sequences are shown in Table 5. The oligonucleotides were made using a PCR mate (Applied Biosystems) and purified in a denaturing acrylamide gel. Details of their purification follow this section.

a. LJC140.

These clones were made by Dr. Chang. Details of the cloning strategy are shown in Figures 14-18. LJC140 was made through a series of sub-clones starting with the creation of pLLL-PAK100 (Figure 14). LJC140 was then made by adding onto the PAK100. PAK100 was cloned into the vector pLLL; details of the oligonucleotides and cloning fragments used are shown in Figure 14. The PAK-100 sequence was excised from pLLL-PAK100 and cloned into the *Bam* HI site of pLLL**gpt** to generate pLLL**gpt**-PAK100 (Figure 15). To complete the LJC140 sequence, two additional complementary oligonucleotides were cloned into the *Avr* II and *Sal* I sites of pLLL**gpt**-PAK100 resulting in the clone pLLL**gpt**-LJC140 (Figure 16).

FIGURE 13. **(a)** Schematic representation of the 5' end of the viral genome, shown in expanded view. Significant landmarks in this region include the 5' copy of the terminal repeat region (R), the unique 5' sequence (U5), the primer binding site for minus stranded DNA synthesis (pbs), dimerization initiation sequence (DIS), the packaging sequence (ψ), the principal splice donor for the formation of the singly and multiply spliced mRNA species (SD) and the beginning of the *gag* gene (AUG in bold type). Nucleotide numbers are relative to the transcription start site at +1. Filled triangle represents 5' CAP structure in the RNA transcript. **(b)** Nucleotide sequence of the untranslated leader from HIV-1 and location of some associated cis-acting elements.

a.


HIV-1 RNA
tat
rev
5'
pol
env
gag
vif
vpu
vpr
nef
(A)n
+1
92
180
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282
346
386
TAR
R
U5
pbs
DIS
ψ
SD
Gag
AUG

b.

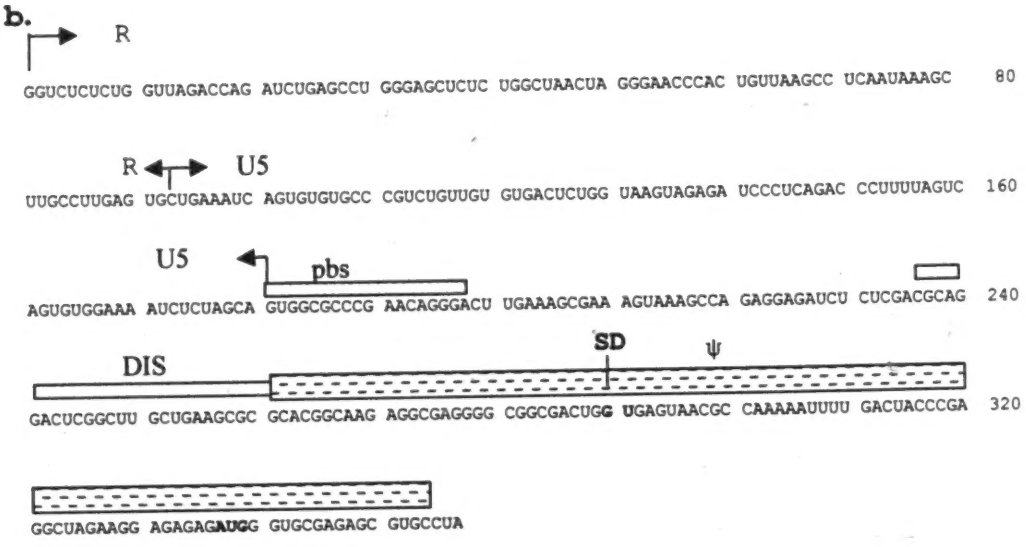

R
GGUCUCUCUG GUUAGACCAG AUCUGAGCCU GGGAGCUCUC UGGCUAACUA GGGAACCCAC UGUUAAGCC UCAAUAAAGC 80
R
U5
UUGCCUUGAG UGCUGAAAUC AGUGUGUGCC CGUCUGUUGU GUGACUCUGG UAAGUAGAGA UCCCUCAGAC CCUUUUAGUC 160
U5
pbs
AGUGUGGAAA AUCUCUAGCA GUGGCGCCCG AACAGGGACU UGAAAGCGAA AGUAAAGCCA GAGGAGAUCU CUCGACGCAG 240
SD
ψ
DIS
GACUCGGCUU GCUGAAGCGC GCACGGCAAG AGGCGAGGGG CGGCGACUGG UGAGUAACGC CAAAAAUUUU GACUACCCGA 320
GGCUAGAAGG AGAGAGAUGG GUGCGAGAGC GUGCCUA

TABLE 5. Oligonucleotides used in the construction of the PAK100, LJC140 and PAK140 elements.

PAK 1	5'-TCGACGGATCCGCAGGACTCGGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGG GCGGCGACTGGCATG-3'
PAK 2	5'-CCAGTCGCCGCCCTCGCCTCTTGCCGTGCGCGCTTCAGCAAGCCGAGTCCTGCG GATCCG-3'
PAK 3	5'-CACGCCAAAAATTTTGACTAGCGGAGGCTAGAAGGAGAGAA AGCTTG-3'
PAK 4	5'-GATCCAAGCTTTCTCTCCTTCTAGCCTCCGCTAGTCAAAATTTTTGGCGTGCATG-3'
anti-gag 1	5'-CTAGACCGGTGCGAGAGCGTCGGTATTAAGCGGGGGAGAAT TACCTAGGTG-3'
anti-gag 2	5'-TCGACACCTAGGTAATTCTCCCCCGCTTAATACCGACGCTCTCGCACCGGT-3'

LJC140 was then cloned into the pLSN and pTAR gene therapy vectors. pLSN-LJC140 was constructed by excising the oligonucleotide generated sequences from pLLL**gpt**-LJC140 using the *Sal* I sites that bracket this area. The fragment generated by this cut was cloned into the *Xho* I site of pLSN (Figure 17). To make pTAR-LJC140, a DNA fragment containing the PAK140 and SV*neo* gene from pLSN-LJC140 was excised by digestion with *Bgl* II and *Sal* I and inserted into pTAR between *Bam* HI and *Sal* I sites (Figure 18).

b. PAK140

The strategy and details of the construction of the PAK140 vectors are shown in Figures 14,15, 19,20, and 21. The strategy used in their creation was similar to that used for the LJC140 series.

3. Oligonucleotide purification

The oligonucleotides used in cloning, the PAK and LJC elements were size purified on a denaturing acrylamide gel. 200 μg (10 μg/μl) of each oligonucleotide was added to 10 μl of stop/loading dye (USB). The oligonucleotide/stop mixture was boiled for 2 min, cooled on ice, and loaded unto a 15 % denaturing acrylamide gel. For a 15 % gel, 75 ml of 30 % acrylamide/1% N,N'-methylenebisacrylamide was mixed with 60 gm of urea in water, the volume was adjusted to 135 ml and 15 ml of 10 times TBE (89 mM Tris base, 89 mM boric acid and 2 mM EDTA) was added to a final volume of 150 ml. Ammonium persulfate (750 μl of a 10% solution in water) and 75 μl of TEMED were added, mixed and poured into a gel. The gel plates were assembled using 5 mm wide spacers. The gel was polymerized for one hour, mounted on a homemade system and electrophoresed for 4-5 hours. Gels were electrophoresed at 800 volts constant power using an ECPS 3000/150 power supply

a.

Fragment A-

Bam HI

tcgacggatccgcaggactcggcttgctgaagcgcgcacggcaagaggcgagggcggcgactggcatg (PAK 1)
gcctaggcgtcctgagccgaacgacttcgcgcgtgccgttctccgctcccgccgctgacc (PAK 2)

Fragment B-

Hind III

cacgccaaaaattttgactagcggaggctagaaggagagaaaagcttg (PAK 3)
gtacgtgcggtttttaaaactgatcgcctccgatcttcctctcttcgaacctag (PAK 4)

(Fragments A and B anneal to create a *Sph* I site[gcatgc].)

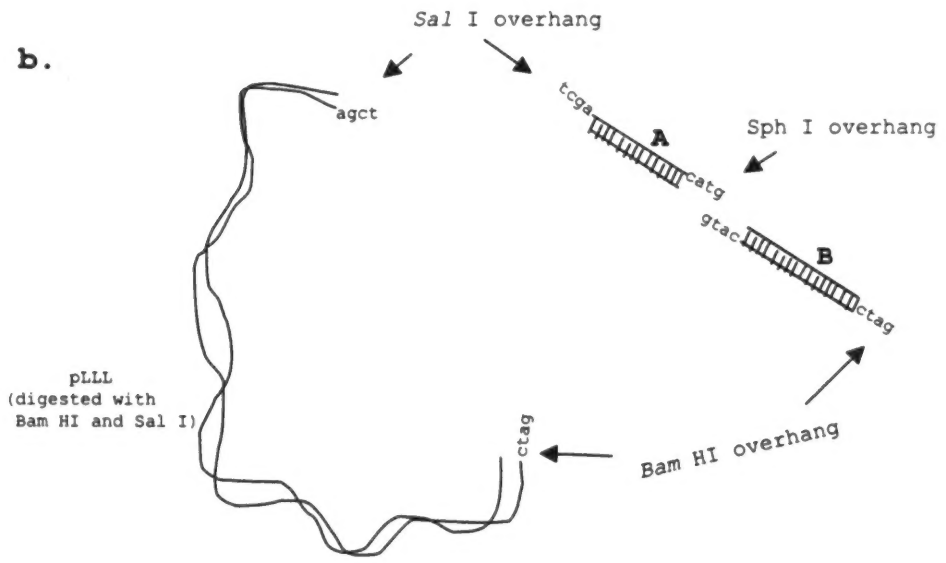
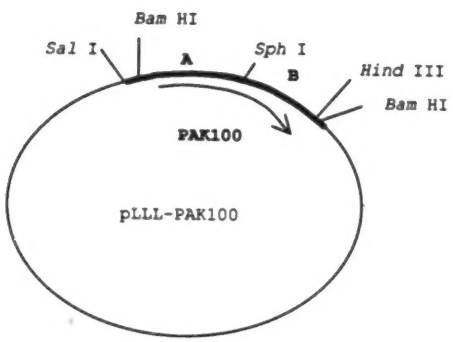
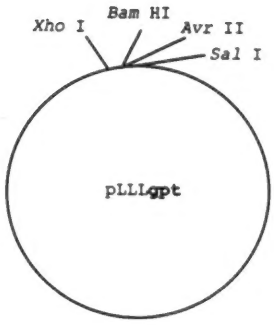
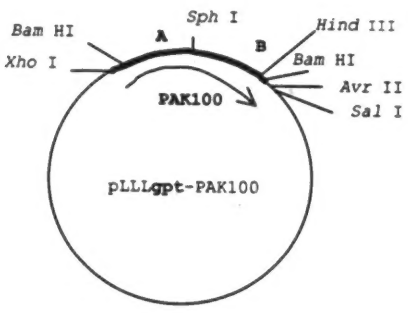


FIGURE 14. Strategy used to clone pLLL-PAK100. (**a**) Annealed oligonucleotides (from Table 5) generate fragments A and B. (**b**) pLLL digested with *Bam* HI and *Sal* I is ligated with fragments A and B.

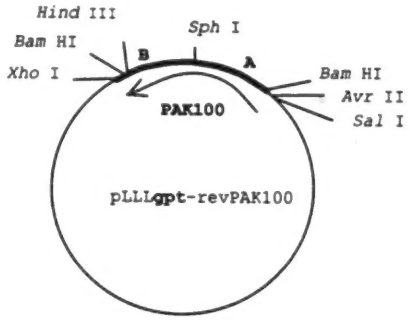




pLLL-PAK100 (see Figure 14b)is cut with *Bam* HI and the resulting 100 basepair fragment is cloned into *Bam* HI digested pLLL**gpt**. This results in the production of two plasmids; pLLL**gpt**-PAK100 and pLLL**gpt**-revPAK100.



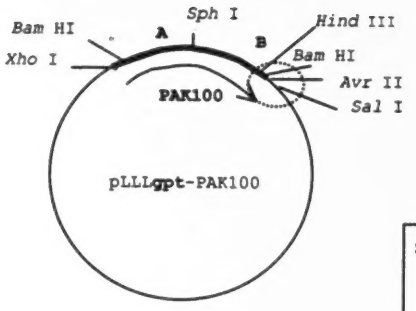
or



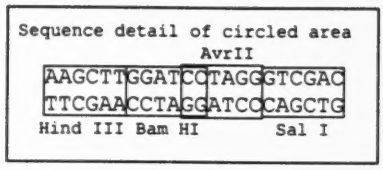
Of these two possible outcomes pLLL**gpt**-PAK100 is used in the construction of pTAR-LJC140

pLLL**gpt**-revPAK100 is used in the construction of pTAR-PAK140

FIGURE 15. Strategy used to clone pLLL**gpt**-PAK100 and pLLL**gpt**-revPAK100.



pLLL**gpt**-PAK100 (see Figure 13) is digested with *Avr* II and *Sal* I.



Oligonucleotides are annealed to generate fragment C.

Fragment C-

Fragment C is ligated into the *Avr* II and *Sal* I sites of pLLL**gpt**-PAK100

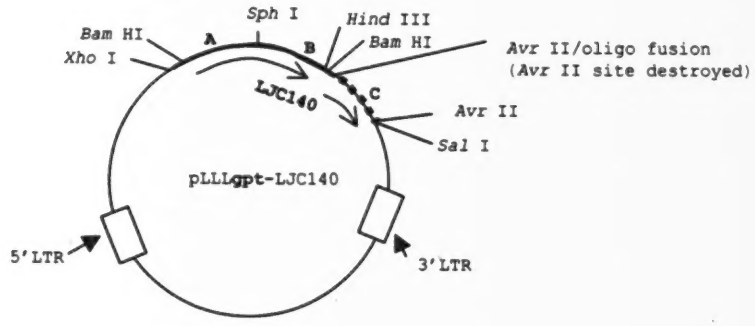
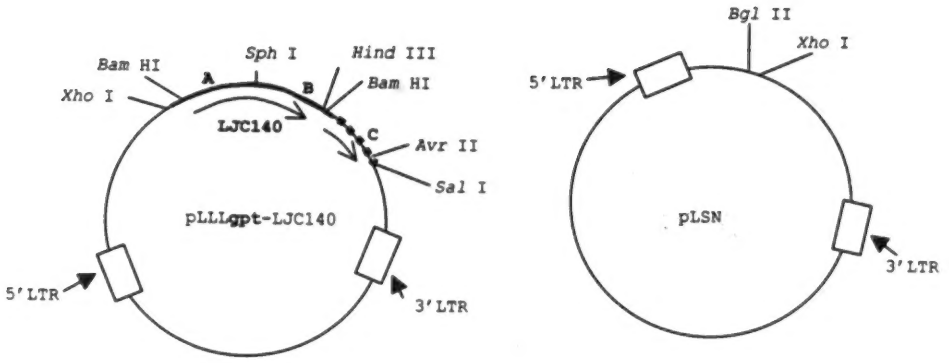


FIGURE 16. Strategy used to clone pLLL**gpt**-LJC140. Note that the original *Avr* II site in the vector is destroyed, but a new *Avr* II site is added within fragment C.



pLLL**gpt**-LJC140(see Figure 14) is digested *Xho* I and *Sal* I to release the LJC140 element. This is cloned into the *Xho* I site of pLSN.

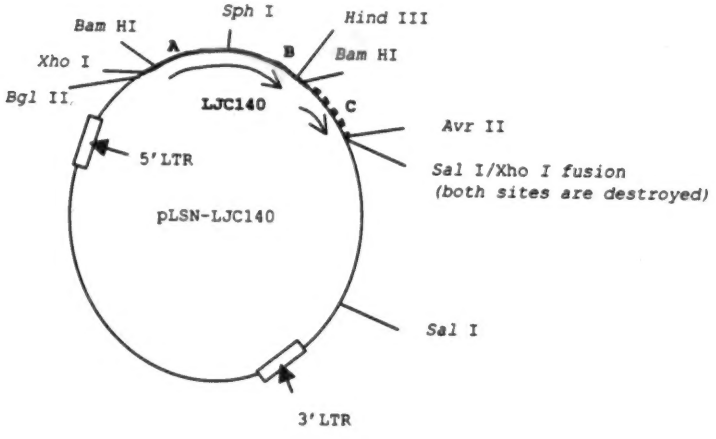
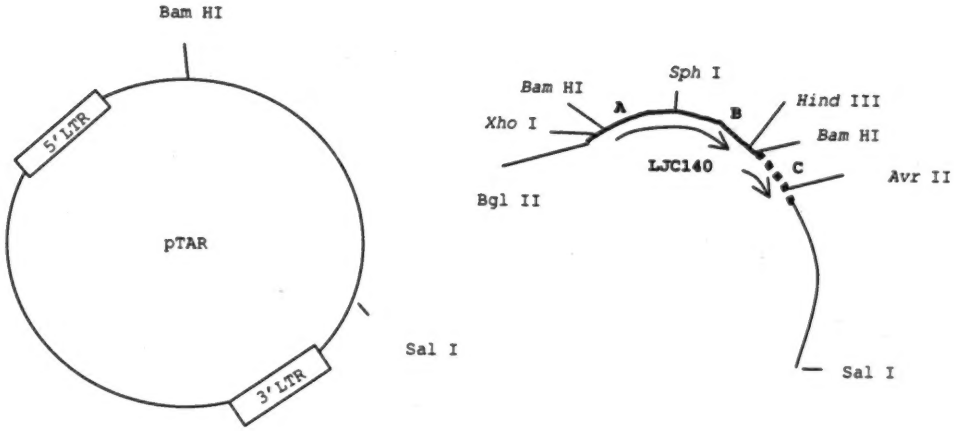


FIGURE 17. Strategy used to clone pLSN-LJC140.



The 1394 bp *Bgl* II-*Sal* I fragment from pLSN-LJC140 (Figure 15) is cloned into the *Bam* HI and *Sal* I sites of pTAR(abbreviated name for the vector pLGCTSN described in Chapter 2).

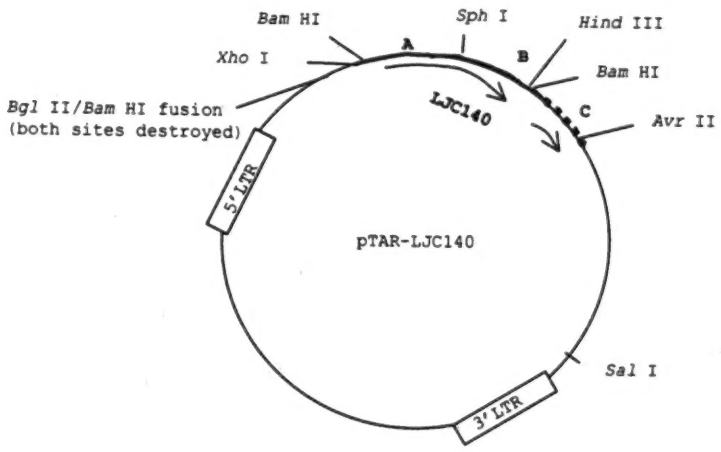
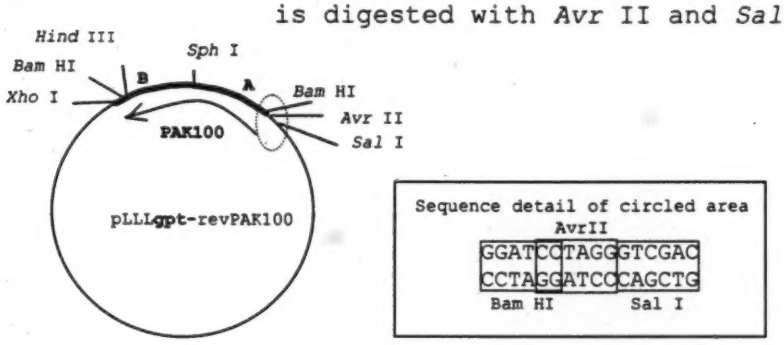


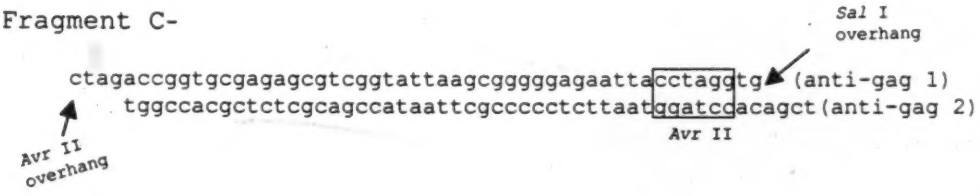
FIGURE 18. Strategy used to clone pTAR-LJC140. Note that in addition to the LJC140 element the 1394 bp fragment contains vector sequences which are exactly the same in both pLSN and pTAR.

pLLL**gpt**-revPAK100 (see Figure 13)
is digested with *Avr* II and *Sal* I.



Oligonucleotides are annealed to generate fragment C.

Fragment C-



Fragment C is ligated into the *Avr* II and *Sal* I sites of pLLL**gpt**-revPAK100

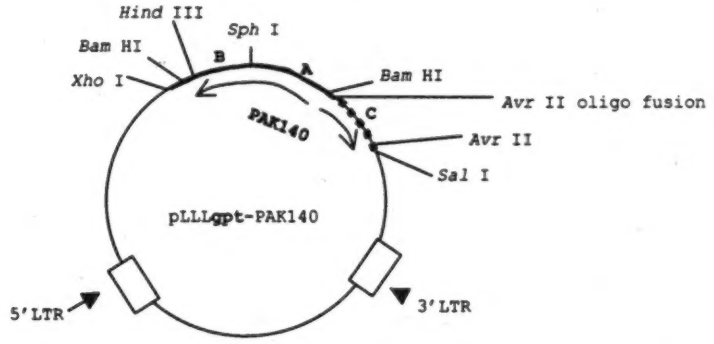
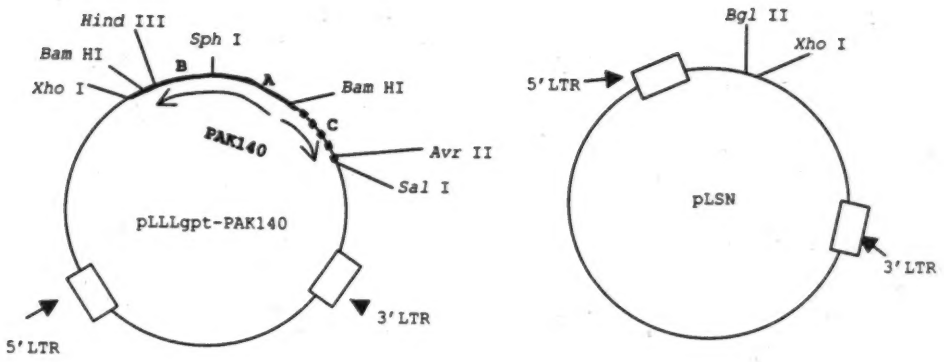


FIGURE 19. Strategy used to clone pLLLgpt-PAK140. As in figure 16 the original *Avr* II site is destroyed, and a new *Avr* II site is added within fragment C.



pLLL**gpt**-PAK140 is cut *Xho* I and *Sal* I to release the PAK140 element. This is cloned into the *Xho* I site of pLSN.

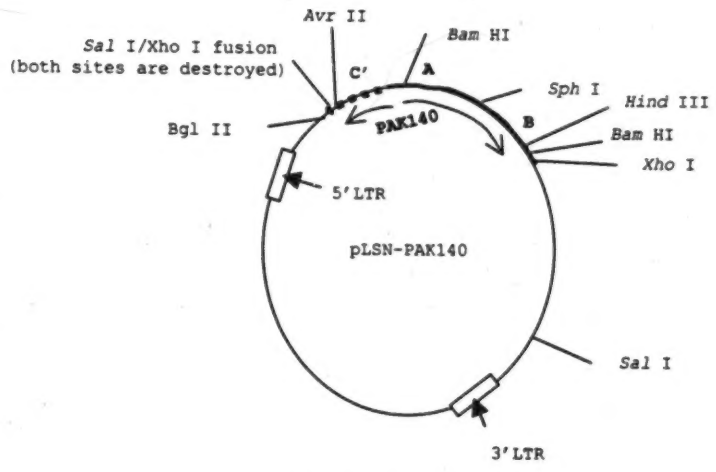
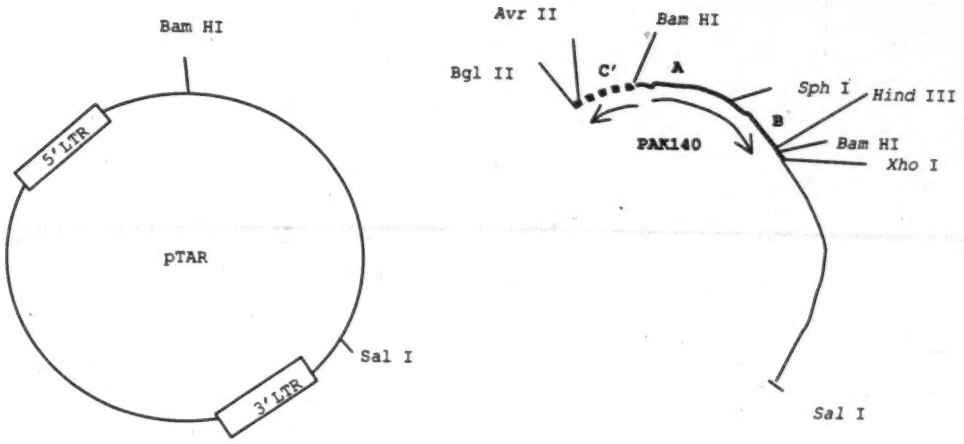


FIGURE 20. Strategy used to clone pLSNPAK-140



The 1394 bp *Bgl* II and *Sal* I fragment from pLSN-PAK140 is cloned into the *Bam* HI and *Sal* I sites of pTAR.

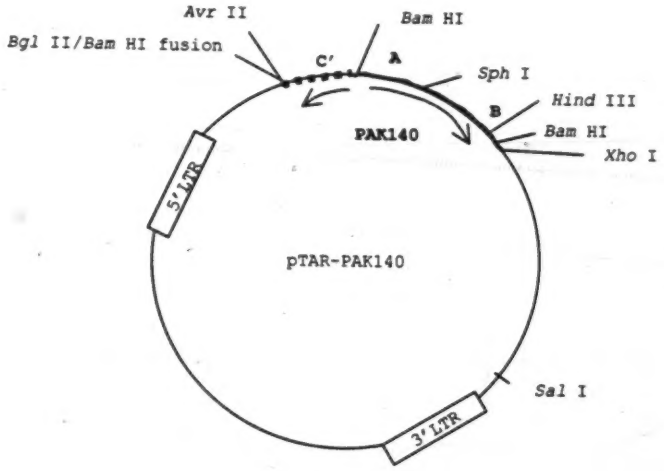


FIGURE 21. Strategy used to clone pTAR-PAK140. Note that in addition to the LJC140 element the 1394 bp fragment contains vector sequences which are exactly the same in both pLSN and pTAR.

(Pharmacia). After running, the gel was removed from the apparatus and the top plate lifted off. The oligonucleotide band could be visualized with an orcinol coated plate and a hand held U.V. light. The band was excised and resuspended in 400 µl of 0.3M NaAcetate pH 7.8. The band was crushed and the slice left to incubate overnight at room temperature with shaking. The next day the liquid was withdrawn from the acrylamide slice and the DNA it contained was precipitated.

4. Cell and virus strains

CEM, CEMTART, H9, C8166 and MT4 were grown in RPMI 1640 containing 10% FBS. The chronic HIV-1 producers MT4.tat+$_{NL4-3}$, MT4.tat-$_{NL4-3}$, and MOLT3.tat+$_{NL4-3}$ were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin. Sources of cell lines are listed in Table 1.

HIV-2$_{CBL-20}$ was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS. HIV-1$_{ELI}$, HIV-1$_{NL4-3}$, and HIV-2$_{ROD}$ was kindly provided by Dr. K. Peden, NIAID, NIH.

5. Kinetic assay for anti-viral effect

H9 is a single cell clone derived from a specific HUT 78 cell line. AA-2 cells are AK-, dCK- subclones, which were derived from the WIL-2 human splenic EBV+ B-lymphoblastoid line. AA-2 cells express high levels of CD4, are remarkably permissive for HIV-1 infection and are extremely sensitive to viral cytopathic effects. The human lymphocytes were infected with HIV-1 or HIV-2 at a multiplicity of infection (MOI) ranging from 0.002-0.2 as indicated in each experiment. The MOI for HIV-2$_{CBL-20}$ was determined

using a $TCID_{50}$ all other MOI's were determined by using reverse transcriptase assays as a measure of the titre of virus.

Culture supernatants were harvested for reverse transcriptase analysis at 3-4 day intervals for the duration of the assay. The harvesting was done by removing 2 of the 5 ml of total culture volume, a small portion of the culture supernatant was retained at -80°C for the RT assay. The cultures were then fed with 2 ml of fresh media. Cells were removed only if the cultures became overgrown. The culture supernatants were frozen at -80°C and the RT assay was done when the samples were complete.

6. Cell viability staining

Cells are mixed in a 1:5 ratio with trypan blue reagent (Gibco, BRL) and counted using a hemacytometer (Hausser Scientific,USA). Cells are considered to be viable if they exclude the blue dye.

7. Reverse Transcriptase Assay

The reverse transcriptase assay was originally described by Goff et al.(138) with modifications introduced by Willey et al (370). 10 µl of culture supernatant was mixed with 50 µl of reaction cocktail. The cocktail contained the following: 50 mM Tris (pH 7.8), 7.5 mM KCL, 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.05% Nonidet P-40, 0.5 µCi of $\alpha^{32}P$ dTTP (Amersham) and a template primer of $(A)n$ (5µg/ml) and $(dT)_{12-18}$. Following a 60-min incubation at 37°C, 3 µl of the reaction mixture was spotted onto DEAE ion-exchange paper (Whatman) and washed three times in 2 times SSC (1 times SSC is 0.15 M NaCl plus 0.015 M sodium citrate pH 7.0). Spots were visualized with a phosphoimager (Fuji) or counted in a scintillation counter (Beckman).

8. Surface analysis of cells expressing CD4

AA-2 or H9 cells were washed twice with IF buffer (PBS with 2% fetal bovine serum and 0.02% sodium azide) and 1 x 10^6 cells were resuspended in 200 µl of IF buffer. The cells were placed on ice for subsequent steps. Cells were stained with anti-CD4-PE (Becton-Dickenson, San Jose, CA, USA) while a second aliquot was stained with the control serum mouse IgG1-PE (Southern Biotechnology Association, Birmingham AL, USA) at a 1:20 dilution for each. Cells were resuspended by flicking tubes and placed on ice for 30 min. After this incubation, cells were centrifuged at 1500 rpm for 5 min, supernatant was removed and cells resuspended in 200 µl of IF buffer as a wash. Cells were washed 2 times more and finally resuspended in 500 µl of PBS with 1% formalin, cells are resuspended and transferred to Falcon 12 x 75 mm polystyrene tubes.

9. Immunostaining of HIV-1 and HIV-2 infected cells

Cells were immunostained as previously described (63) with the following modifications: The infected cells were washed with cold PBS, allowed to attach to a poly-D-lysine coated plate for 10 min and washed with PBS twice more. The attached cells were then fixed with 1:1 methanol:acetone at -20°C for 3 min and incubated with pooled HIV-1 or HIV-2 patients' sera at 1:1000 dilution at 4°C overnight. The pooled anti-HIV-1 sera were obtained from the HIV Clinic in Edmonton AB Canada. The pooled anti-HIV-2 sera were provided by the AIDS Research and Repository Program of NIH, USA. After the first antibody, the cells were blocked with normal sheep serum prior to the addition of the secondary biotinylated sheep anti-human antibody (Amersham). The cells were incubated with the secondary antibody for 1 h at room temperature, washed 4-5 times, then followed

by incubation with avidin and biotinylated horseradish peroxidase (Pierce Chemical Co.'s ABC reagent, USA) for 30 min at room temperature. The peroxidase reaction was developed with 3-3'-diaminobenzidine tetrahydrochloride solution (Sigma's DAB) and 0.3% $NiCl_2$ for 3-4 min and stopped by rinsing cells with water for 1-2 min. The positively stained cells were counted under an inverted microscope.

10. Retroviral transduction

The gene therapy constructs were transfected by lipofection into packaging cell line PA317, and the culture supernatant was collected after 48 h and used for transduction (290). Human lymphoid cell lines AA-2, H9, or MT4 (2×10^6 cells) were incubated with supernatant collected from the transfected PA317 cells in the presence of 4-8 μg/ml of polybrene (Sigma). Twenty-four hours after transduction, G418 at a final concentration of 1 mg/ml was added to the culture and G418-resistant cells were obtained 20 days later.

11. Northern blot

This method is a variation on the one described previously with the following changes: Total poly A+ RNA was harvested as described (18) with minor modifications. Cells were pelleted, washed with PBS and lysed in 1 ml proteinase K buffer (0.5 M NaCl, 1% SDS, 10 mM Tris pH 7.5 and 1 mM EDTA). Chromosomal DNA was sheared by pulling the DNA through a 25 gauge needle 3 times and the resulting solution was digested at 37°C for 30 min with 100 μg/ml proteinase K. After digestion, the polyA+ RNA was selected by adding 30 μl of oligo-dT cellulose powder (Collaborative Research) and mixing at room temperature for 1 h. Oligo-dT was pelleted and washed three times with 1 ml of the proteinase K buffer and one time with the same buffer containing only 0.1 M NaCl. The

polyA+ RNA was eluted twice in 200 µl of T.E. (10 mM Tris-HCl pH 7.5, 1mM EDTA) and precipitated by adding 40 µl of 5 M ammonium acetate and 1 ml of 95% ethanol. The RNA was analyzed by electrophoresis on a formaldehyde agarose gel pH 7.8, blotted on to a Nitrocellulose membrane and probed with random primed probes (Stratagene) using sequences specific for PAK140, SVneo, or ß-actin as previously described (62). For the blots that were used more than once, the old probe was stripped by boiling the blots in ddH_2O containing 0.01% SDS for 10 min.

C. RESULTS

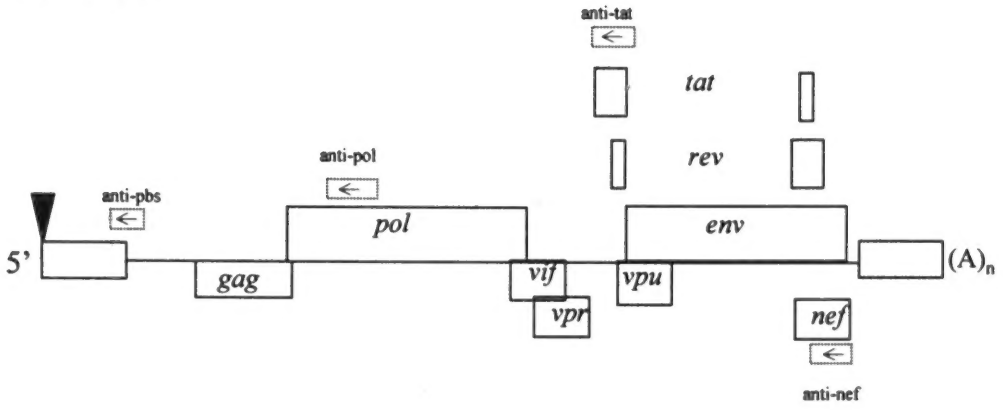
1. Initial antisense constructs did not inhibit HIV virus production in a HeLa cell co-transfection assay

Four different antisense proviral plasmids were constructed, containing sequences targeting various regions of the HIV-1 virus genome. These included the primer binding site (pLSNanti-pbs), the tat gene (pLSNanti-tat), the pol gene (pLSNanti-pol) and the nef gene (pLSNanti-nef) (Figure 22a). In order to assess their potential antiviral effect, each of the antisense plasmids was transfected into HeLa cells, together with the plasmid pNL4-3. pNL4-3 contains the entire HIV proviral genome and when transfected alone into HeLa cells generates infectious HIV virus. Since the intact virions contain reverse transcriptase, the quantity of virus present in the supernatant can be assayed indirectly by measuring RT

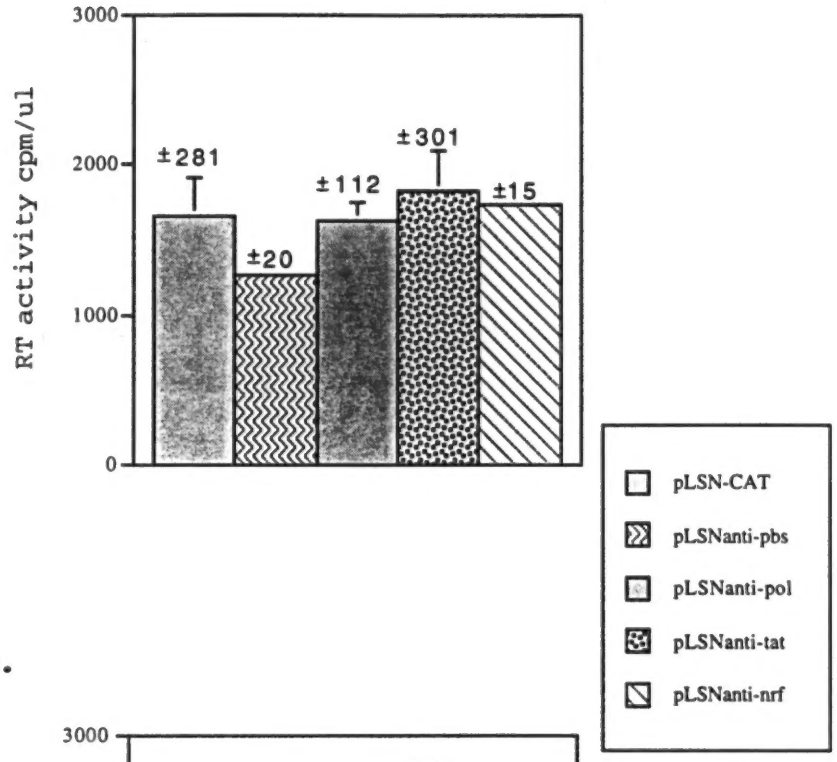
FIGURE 22. Virus production from HeLa cells transfected with an HIV-1 proviral plasmid (pNL4-3) together with various antisense constructs.(**a**) Shows a schematic of the approximate size and position of the various antisense transcripts tested. (**b**) Molar ratio of antisense plasmid to HIV plasmid is 2:1. (**c**) Molar ratio of antisense plasmid to HIV plasmid is 4:1. (Supernatants were harvested at 72 h and assayed for reverse transcriptase (RT). Virus production was assumed to be proportional to RT activity). When transfected into HeLa cells pNL4-3 generates infectious virus in the culture supernatant; successful antisense constructs would be expected to reduce virus production by at least 10 fold.

a.

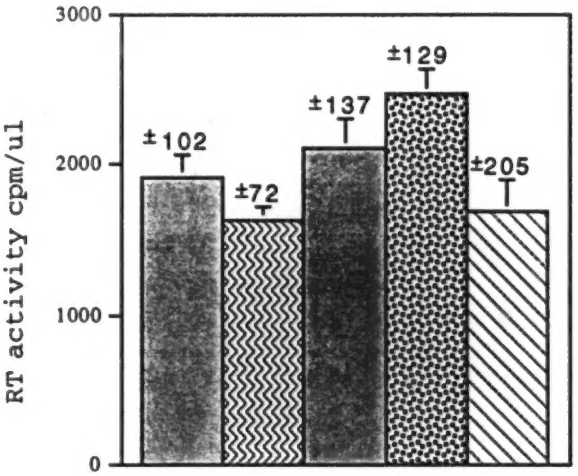
HIV-1 RNA


anti-tat
tat
rev
anti-pol
anti-pbs
pol
env
5'
gag
vif
vpu
vpr
nef
(A)n
anti-nef

b.


3000
2000
1000
0
RT activity cpm/ul
±281
±20
±112
±301
±15
pLSN-CAT
pLSNanti-pbs
pLSNanti-pol
pLSNanti-tat
pLSNanti-nrf

c.


3000
2000
1000
0
RT activity cpm/ul
±102
±72
±137
±129
±205

activity. As shown in Figure 22, the quantity of HIV virus produced form HeLa cells co-transfected with a control plasmid (pLSN-cat) was essentially the same as that produced when any one of the antisense constructs was co-transfected. This was true when the plasmids were co-transfected at a molar ratio of 2:1 or 4:1 (antisense:pNL4-3). Given this initial result we chose not to pursue this strategy any further, but rather went on to explore a strategy based on a decoy packaging signal.

2. Two different HIV packaging decoy constructs were created: LJC140 (all sense strand) and PAK140 (mixture of sense and antisense).

The leader and early gag regions of HIV contain critical packaging signals (Figure 13). Originally we intended to generate a 140 bp DNA element which would give rise to an entirely sense strand HIV packaging decoy. However, through a cloning error we actually ended up generating two different forms of the 140 bp construct, named LJC140 and PAK140. The precise origins of the two constructs are described in the Materials and Methods section of this chapter; Figure 23 compares them directly. The major difference between the two constructs is that PAK140 contains a 5' segment of gag which is antisense, and therefore capable of hybridizing to HIV proviral transcripts (Figure 23c). The experimentally determined DNA sequences of the 100 bp intermediate construct (PAK100) as well as LJC140 and PAK140 are shown in Figure 24. All of our initial in vitro experiments utilized PAK140.

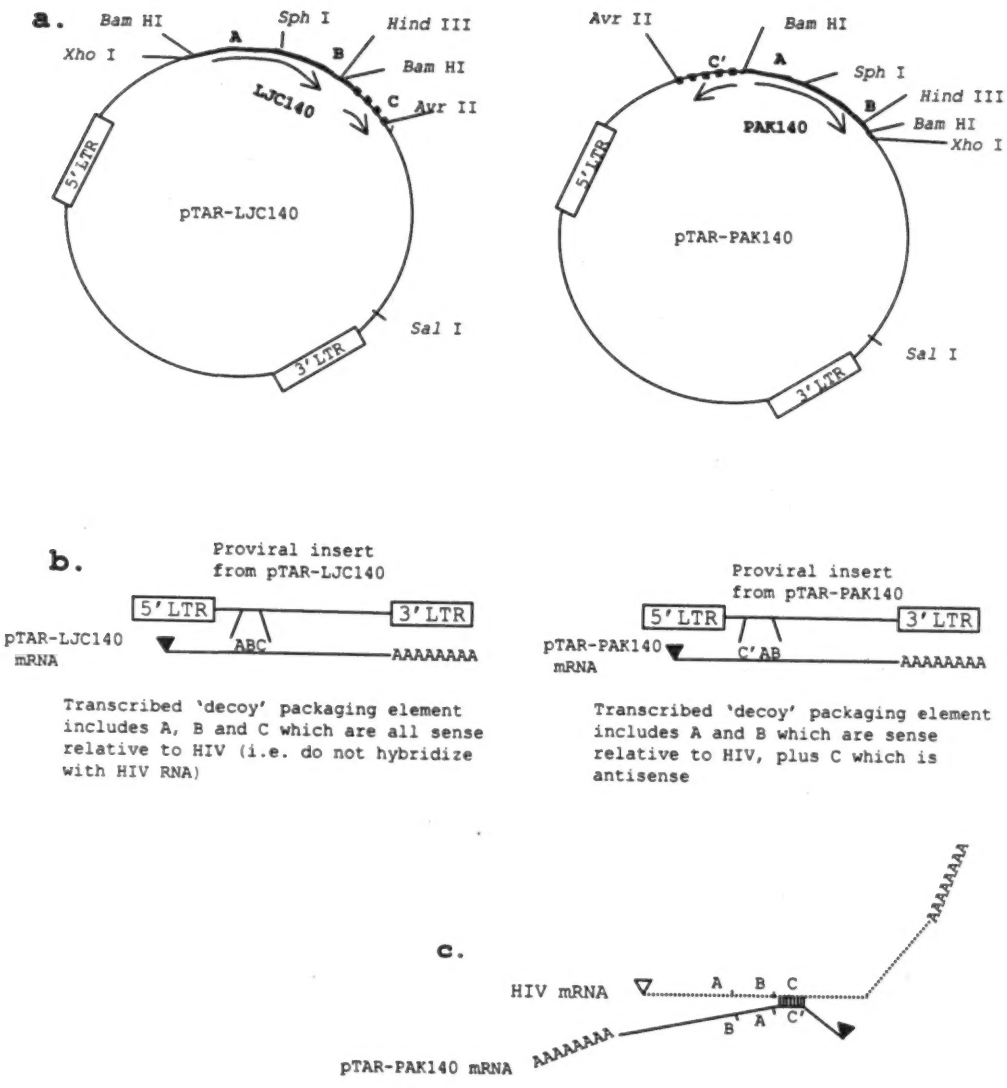
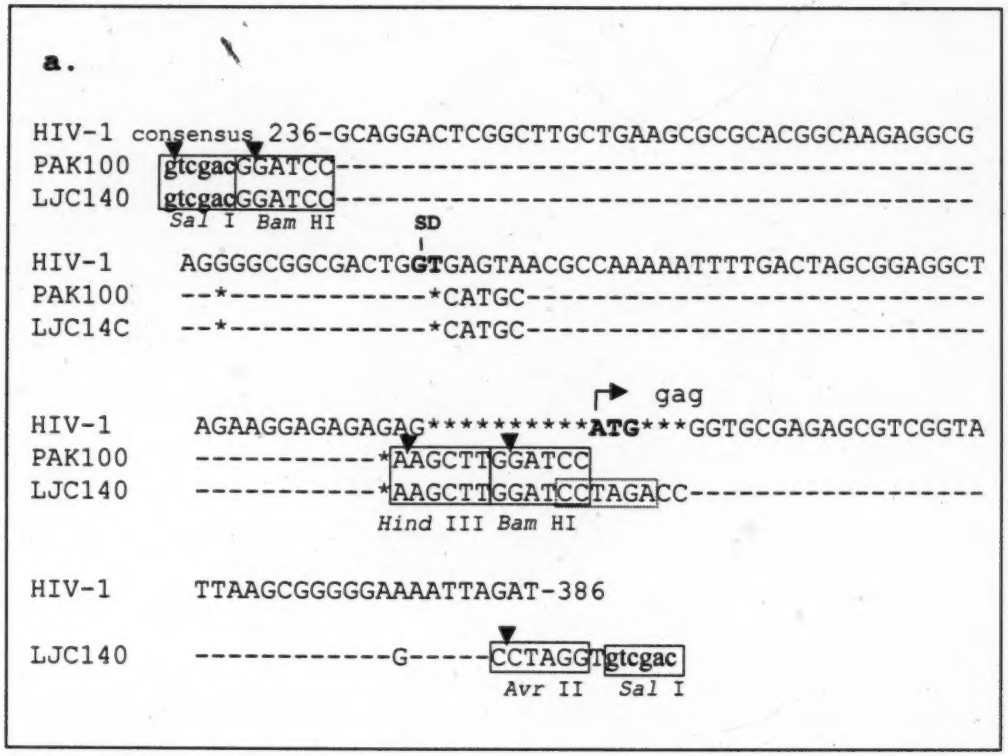
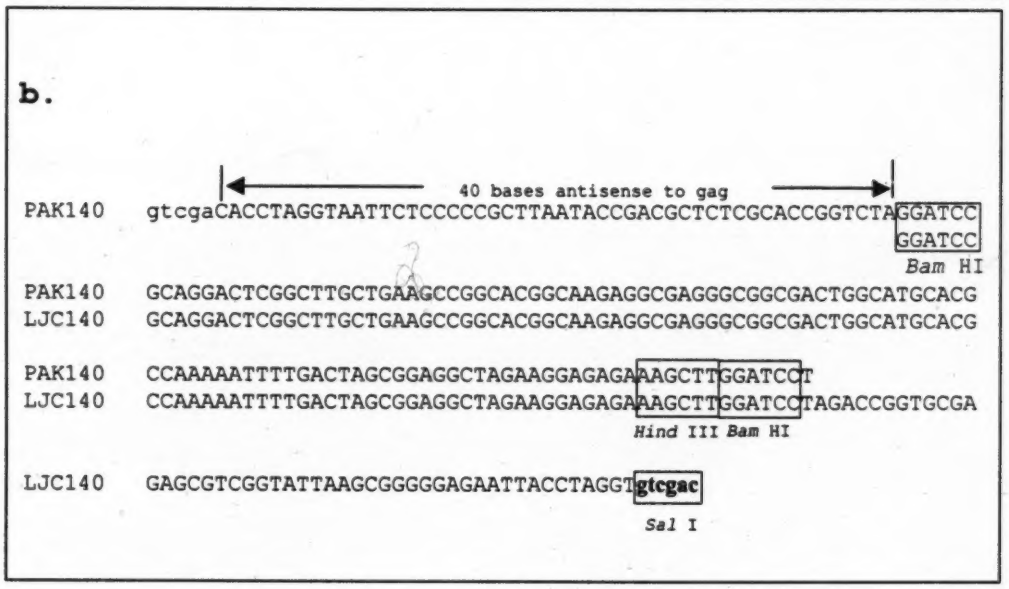


FIGURE 23. (**a**) Comparison of pTAR-LJC140 and pTAR-PAK140 constructs. (**b**) Comparison of predicted mRNAs transcribed from constructs pTAR-LJC140 and pTAR-PAK140. (**c**) The transcript from pTAR-PAK140 is capable of annealing with the HIV-1 transcript (dotted line) as shown.

FIGURE 24. (**a**) DNA sequences of the synthetic HIV-1 packaging elements PAK100 and LJC140. The dashed lines indicate regions where the sequence is identical to the HIV-1 consensus. The sequences differ from the HIV consensus at the major splice donor and at the gag initiation codon. Numbering is relative to the transcriptional start site as in Figure 11. Restriction sites are outlined in solid boxes, and the dotted box indicates the *Avr* II/Fragment C fusion (see Figure 14). *Sal* I sites used in the original cloning are indicated in lower case times font; note that the 5' *Sal* I site is not carried through to the LSN or TAR constructs. (**b**) Comparison of the sequences of LJC140 and PAK140. The PAK100, LJC140 and PAK140 sequences in **a** and **b** were all confirmed by DNA sequencing.


a.
HIV-1 consensus 236-GCAGGACTCGGCTTGCTGAAGCGCGCACGGCAAGAGGCG
PAK100 gtcgacGGATCC----------
LJC140 gtcgacGGATCC----------
Sal I Bam HI
SD
HIV-1 AGGGGCGGCGACTGGTGAGTAACGCCAAAAATTTTGACTAGCGGAGGCT
PAK100 --*----------*CATGC----------
LJC14C --*----------*CATGC----------
gag
HIV-1 AGAAGGAGAGAGAG**********ATG***GGTGCGAGAGCGTCGGTA
PAK100 ----------*AAGCTTGGATCC
LJC140 ----------*AAGCTTGGATCCTAGACC----------
Hind III Bam HI
HIV-1 TTAAGCGGGGGAAAATTAGAT-386
LJC140 ----------G-----CCTAGGTgtcgac
Avr II Sal I


b.
40 bases antisense to gag
PAK140 gtcgaCACCTAGGTAATTCTCCCCCGCTTAATACCGACGCTCTCGCACCGGTCTAGGATCC
GGATCC
Bam HI
PAK140 GCAGGACTCGGCTTGCTGAAGCCGGCACGGCAAGAGGCGAGGGCGGCGACTGGCATGCACG
LJC140 GCAGGACTCGGCTTGCTGAAGCCGGCACGGCAAGAGGCGAGGGCGGCGACTGGCATGCACG
PAK140 CCAAAAATTTTGACTAGCGGAGGCTAGAAGGAGAGAAAGCTTGGATCCT
LJC140 CCAAAAATTTTGACTAGCGGAGGCTAGAAGGAGAGAAAGCTTGGATCCTAGACCGGTGCGA
Hind III Bam HI
LJC140 GAGCGTCGGTATTAAGCGGGGGAGAATTACCTAGGTgtcgac
Sal I

3. A human lymphoblastoid cell line stably transduced with TAR-PAK140 gave rise to reduced levels of virus following HIV-1 infection.

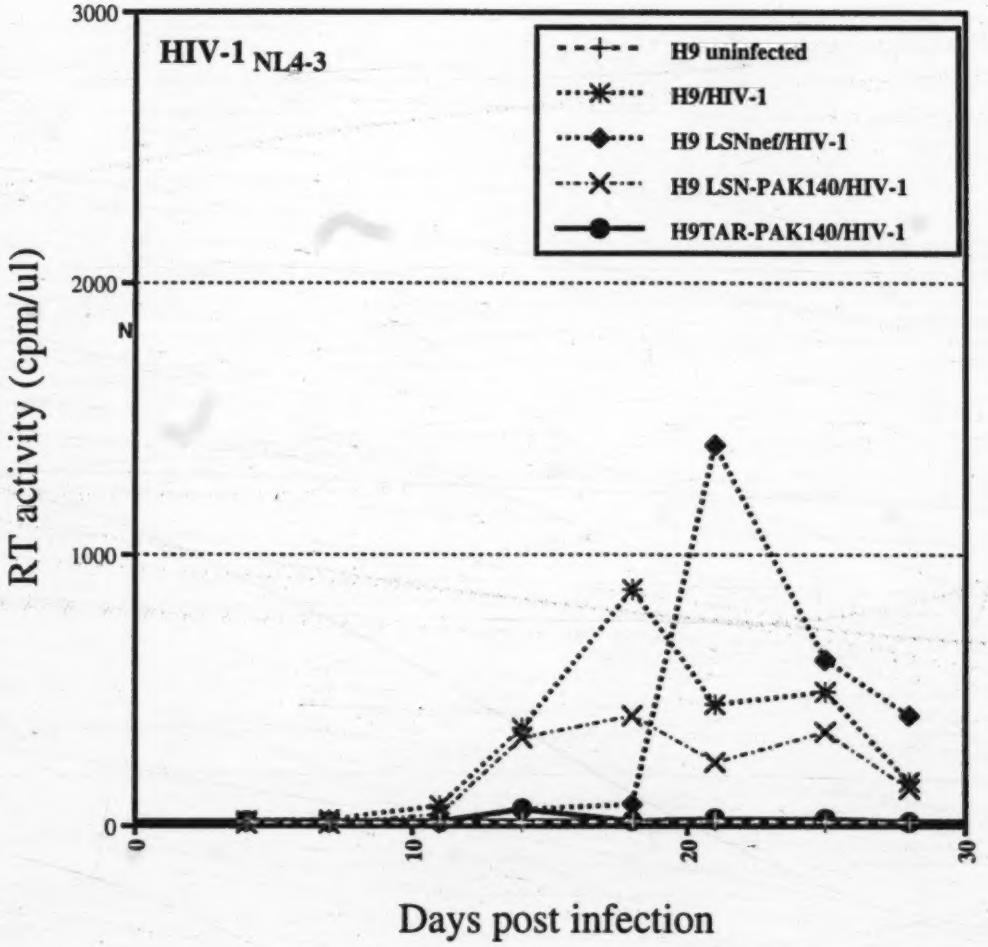
The PAK140 element was inserted into two different retroviral vectors, pLSN, and pTAR, and both of these were transiently transfected into the amphotrophic packaging cell line PA317. Culture supernatants from the PA317 cells were collected 72 h after transfection, and provirus from these harvests were demonstrated to transfer neomycin resistance to a number of cell lines. This allowed us to proceed with model in vitro experiments using two CD4+, HIV susceptible human cell lines; H9 and AA-2. Both cell lines are extremely sensitive to HIV-1 infection and to the cytopathic effects of the virus.

H9 and AA-2 cells were both transduced with three different retroviruses (LSNnef, LSN-PAK140, and TAR-PAK140), and G418 selection was used to establish six stable transduced cell lines. The parental cells and each of the transduced lines were then challenged with HIV-1_{NL4-3} at low MOIs. Progress of the HIV infection in all cell lines was followed by taking serial samples of culture supernatant (at days 3,7,11,14,17,21,24,28, and in some cases also days 31,35,38,40), storing these at -80°C, and assaying for RT activity once all the samples had been collected. Typical results obtained are shown in Figure 25 and 26 for H9 cells, and Figure 27 for AA-2 cells.

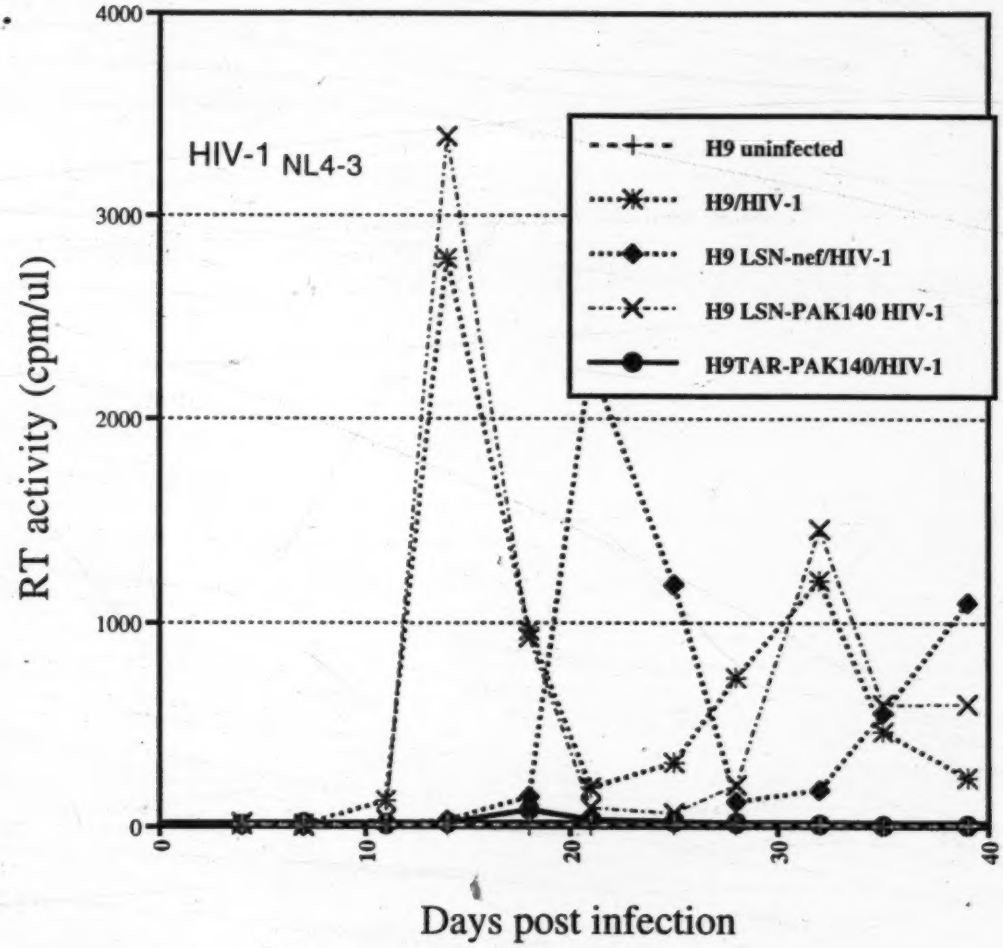
In comparison to the other transduced H9 cell lines, H9-TAR-PAK140 cells gave markedly diminished (although not completely abolished) virus production following HIV-1 infection at a MOI of 0.001 (Figure 25a) or 0.002 (Figure 25b). A possible explanation for these results is that TAR-PAK140 transduced H9 cells died rapidly following viral challenge, and therefore were unable to produce any virus after the initial infection. However, Figure

FIGURE 25. Kinetics of HIV virus production in wild-type and transduced H9 cells infected with HIV-1_{NL4-3}. H9 cells were transduced with the recombinant retroviruses LSN-nef, LSN-PAK140 or TAR-PAK140 and stable cell lines were established using G418 selection. Equal numbers of wild-type or transduced H9 cells were seeded into identical flasks (2 X 10^6 cells/25 cm^2/5 ml of media) and then were infected with HIV-1_{NL4-3} at time zero. Aliquots of culture supernatant (2 ml) were harvested from each flask at 3-4 day intervals, and the volume removed was replaced with fresh media each time. Supernatants were stored at –80°C and an RT assay performed on all samples at the end of each study. RT assays were done in duplicate for each sample, and average values are shown; the duplicates typically gave values within 1% of each other. Average RT activities for various culture supernatants are plotted versus time, using an initial MOI of 0.001 (**a**) or 0.002 (**b**). In the experiment shown in b the number of live cells present in each culture flask at various times post infection was determined using trypan blue staining, results are shown in (**c**). Note that in (c) the number of live cells is expressed as a percentage of the number of live cells present in the uninfected H9 culture flask. At 7 or 14 days post-infection, cells from cultures set up in parallel to the experiment shown in (b) were harvested, attached to poly-D-lysine coated 6-well plates, fixed with methanol/acetone, and immuno-peroxidase stained for the presence of HIV antigens. Representative photomicrographs of the immunostained cells are shown in figures (**d**) and (**h**), (uninfected H9 cells), (**e**) and (**i**), (H9 cells infected with HIV-1_{NL4-3}), (**f**) and (**j**), (H9 cells transduced with LSN-PAK140 and infected with HIV-1_{NL4-3}) and (**g**) and (**k**), (H9 cells transduced with TAR-PAK140 and infected with HIV-1_{NL4-3}). The experiments in (b), (c) and (d) was repeated a second time, and gave a very similar result (data not shown). In this series of experiments the H9 cells transduced with LSN-nef represent a second negative control; this provirus contained part of the HIV-1 nef gene in the sense orientation, driven by the same MLV LTR promoter present in LSN-PAK140.

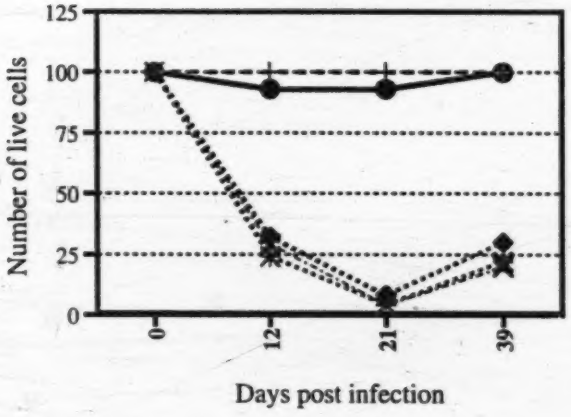
a.


HIV-1 NL4-3
H9 uninfected
H9/HIV-1
H9 LSNnef/HIV-1
H9 LSN-PAK140/HIV-1
H9TAR-PAK140/HIV-1
RT activity (cpm/ul)
3000
2000
1000
0
0
10
20
30
Days post infection

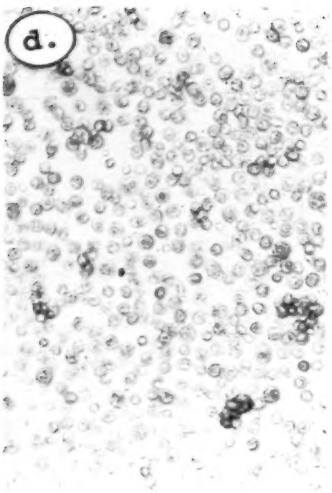
b.

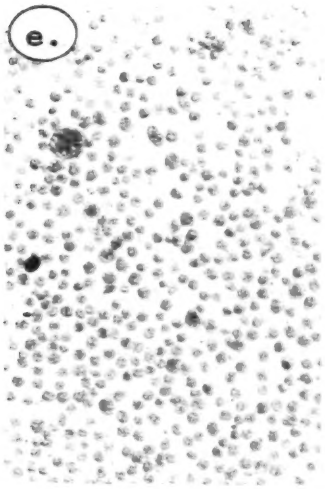

HIV-1 NL4-3
H9 uninfected
H9/HIV-1
H9 LSN-nef/HIV-1
H9 LSN-PAK140 HIV-1
H9TAR-PAK140/HIV-1
RT activity (cpm/ul)
4000
3000
2000
1000
0
0
10
20
30
40
Days post infection

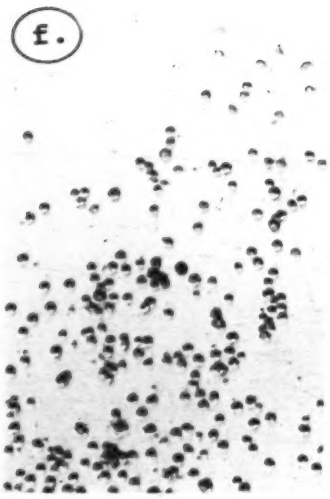
c.

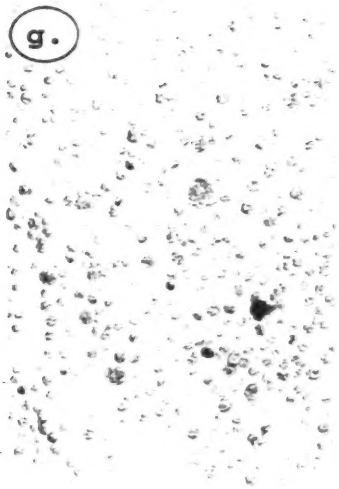

Number of live cells
125
100
75
50
25
0
0
12
21
39
Days post infection

DAY SEVEN







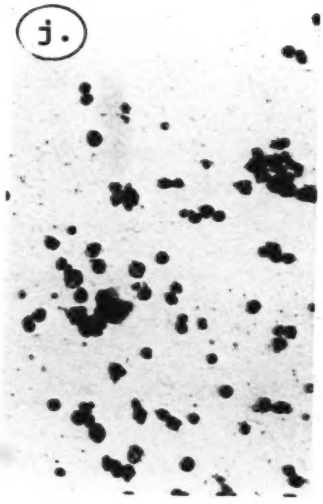


DAY FOURTEEN

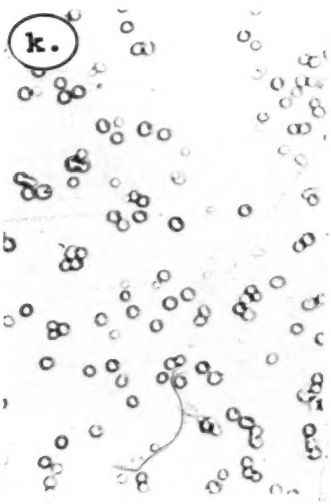
h.

i.

j.



k.



25c shows that this was not the case, since the number of live cells present throughout the experiment remained almost the same as in the uninfected control cultures. Figures 25d through 25k show the immunostained cells at days 7 or 14 post-HIV infection. Although the day 7 cells all look very similar, by day 14 the untransduced and LSN-PAK140 transduced cell lines are heavily infected with HIV-1 virus, whereas the TAR-PAK140 transduced cells appear similar to the uninfected cells

Given that our initial results were obtained with a single strain of HIV-1 (NL4-3), we went on to infect our H9 transduced cells with a second virus, HIV-1_{ELI}. Since HIV-1_{ELI} is not as cytopathic in tissue culture, a higher MOI (0.2) was used. These results (Figure 26) again show that the H9-TAR-PAK140 transduced cells did not produce as much virus as the other transduced lines. The peak RT levels are reduced about 6 fold, and the peak occurs about 10 days later.

The results obtained with the AA-2 transduced cell lines are qualitatively similar (Figures 27a and 27b; both using a MOI of 0.002), although it is more apparent in these experiments that the TAR-PAK140 transduced cells do produce HIV-1 virus post-infection (up to about 25% of control levels; Figure 27a). Consistent with this fact is the observation that the HIV infected, TAR-PAK140 transduced AA-2 cultures did show reduced numbers of viable cells (Figure 27c) and significant numbers of infected cells (Figure 27g).

In a final series of experiments the H9 transduced cell lines were challenged with HIV-2 (Figure 28). Similar to the results obtained when the transduce H9 cells were challenged with HIV-1, challenge with HIV-2_{ROD} gave markedly reduced virus production in the TAR-PAK140 transduce cells. Reduced virus production (25% of control) was also

FIGURE 26. Kinetics of HIV virus production in wild-type and transduced H9 cells infected with HIV-1_{ELI}. This experiment is identical to that described in Figure 23, except that the MOI is increased to 0.2. Note that in comparison to HIV-1_{NL4-3}, HIV-1_{ELI} is known to be less cytopathic in tissue culture.

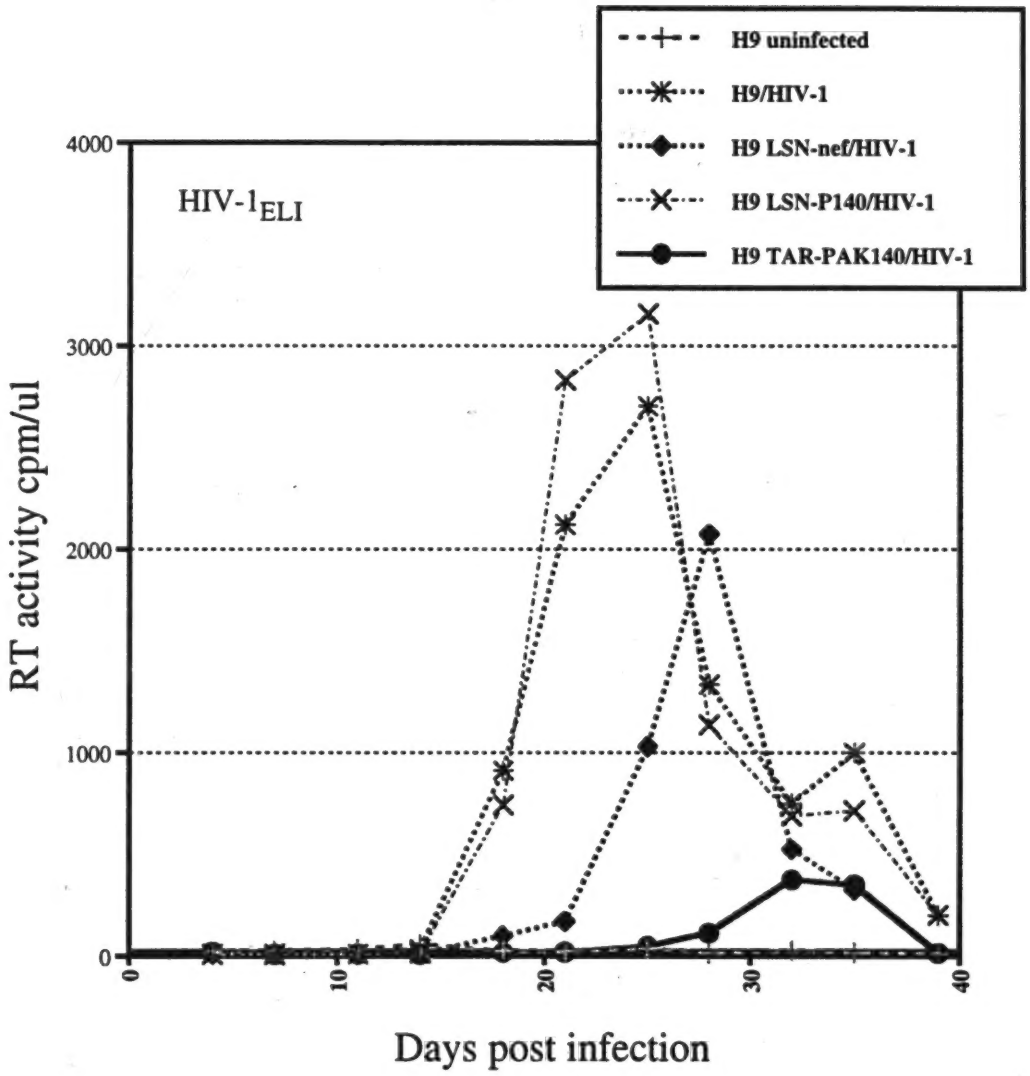
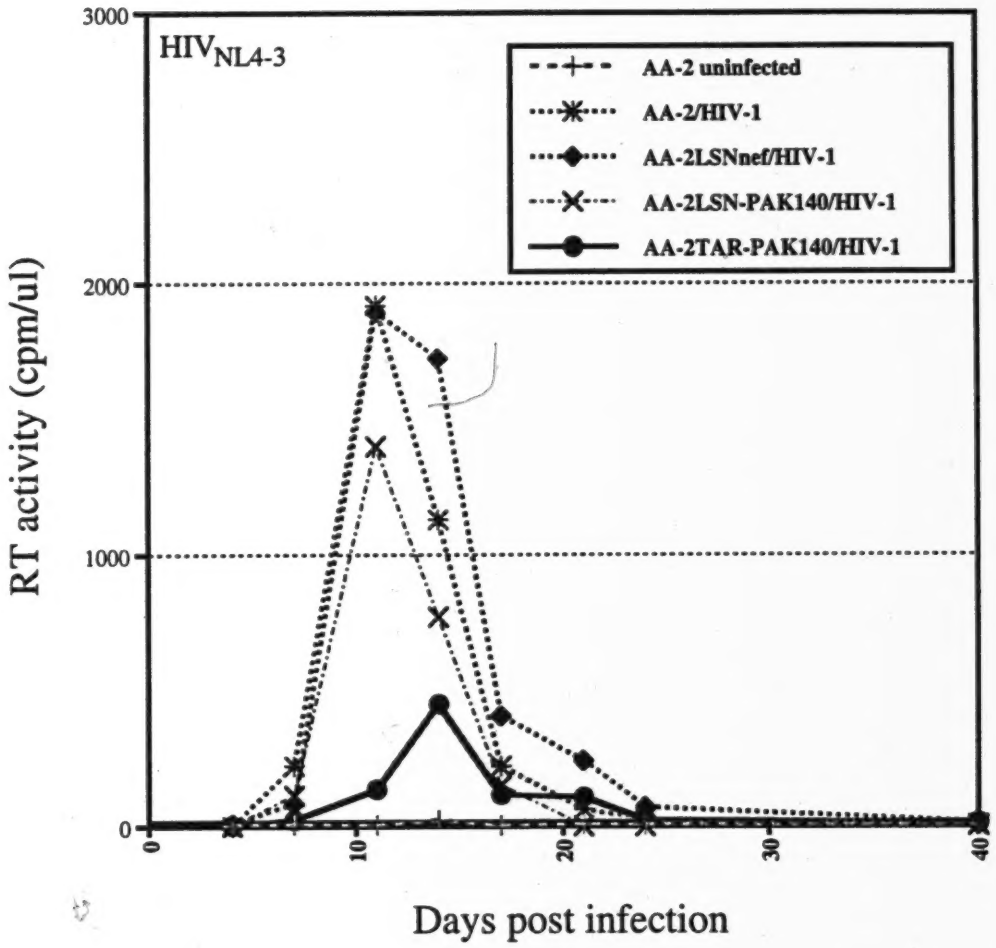
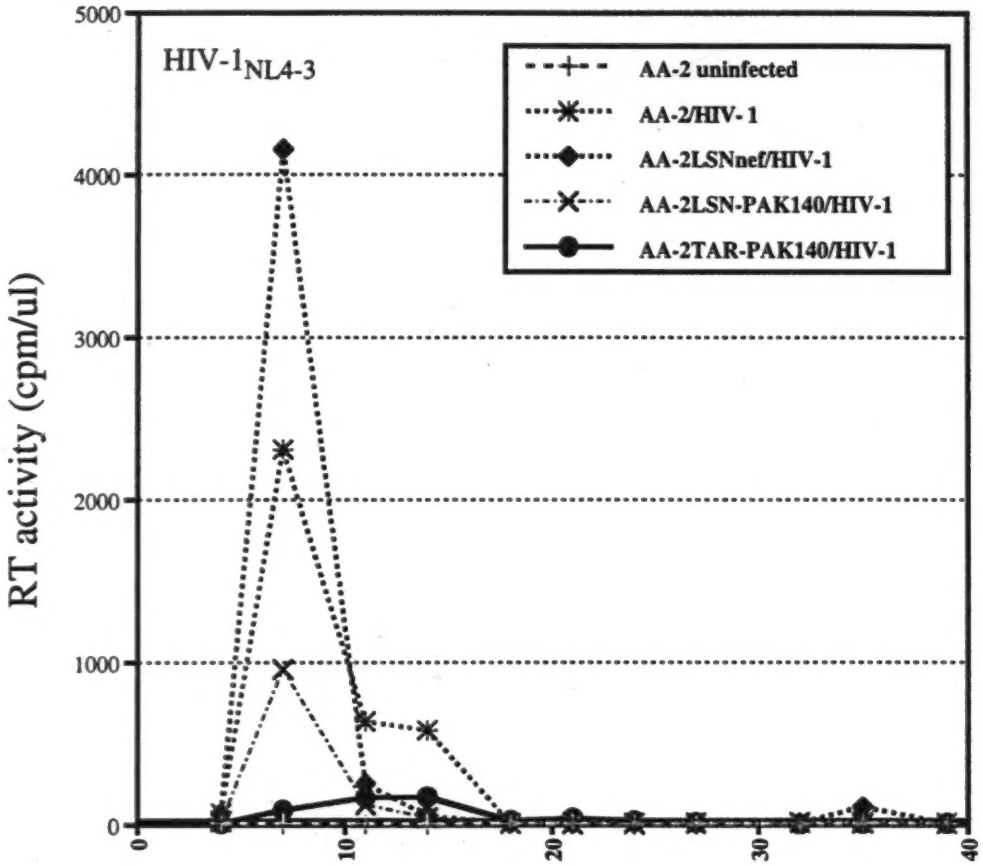

H9 uninfected
H9/HIV-1
H9 LSN-nef/HIV-1
H9 LSN-P140/HIV-1
H9 TAR-PAK140/HIV-1
HIV-1ELI
RT activity cpm/ul
4000
3000
2000
1000
0
0
10
20
30
40
Days post infection

FIGURE 27. Kinetics of HIV virus production in wild-type and transduced AA-2 cells infected with HIV-1_{NL4-3}. AA-2 cells were transduced with the recombinant retroviruses LSN-nef, LSN-PAK140 or TAR-PAK140 and stable cell lines were established using G418 selection. Equal numbers of wild-type or transduced AA-2 cells were seeded into identical flasks (2 X 10^6 cells/25 cm^2/5 ml of media) and then were infected with HIV-1_{NL4-3} at time zero. Aliquots of culture supernatant were harvested and assayed for RT as in Figure 23. This experiment was repeated three times, each time using an MOI of 0.002 and results of two of the experiments were shown in (**a**) and (**b**). In the second experiment the number of live cells present in each culture flask at various times was determined using trypan blue staining (**c**). Note that again the number of live cells is expressed as a percentage of the number of cells present in the uninfected AA-2 culture. At 7 days post-infection, cells from cultures set up in parallel to the experiment shown in (a) were harvested and immunostained for the presence of HIV antigens. Representative photomicrographs of the immunostained cells are shown in figures (**d**), (uninfected AA-2 cells), (**e**), (AA-2 cells infected with HIV-1_{NL4-3}), (**f**), (AA-2 cells transduced with LSN-PAK140 infected with HIV-1_{NL4-3}) and (**g**), (Aa-2 cells transduced with TAR-PAK140 and infected with HIV-1_{NL4-3}).

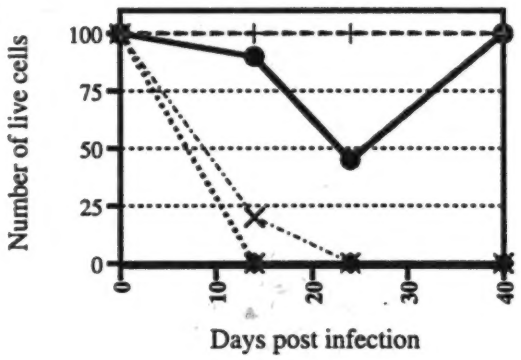
a.


HIV$_{NL4-3}$
AA-2 uninfected
AA-2/HIV-1
AA-2LSNnef/HIV-1
AA-2LSN-PAK140/HIV-1
AA-2TAR-PAK140/HIV-1
RT activity (cpm/ul)
3000
2000
1000
0
0
10
20
30
40
Days post infection

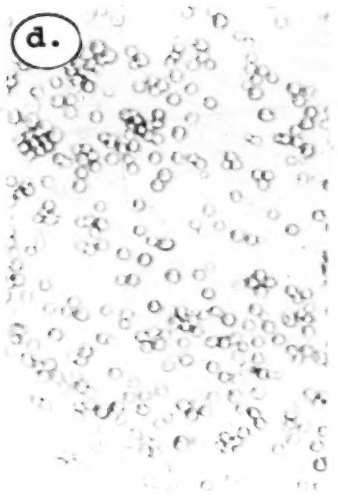
b.

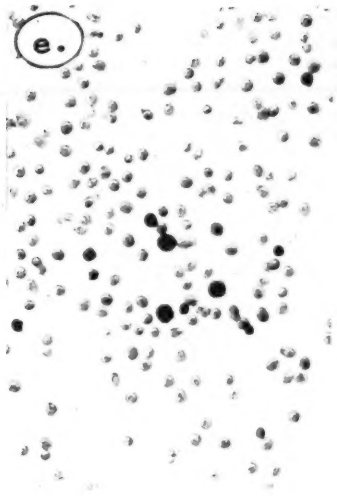

HIV-1NL4-3
AA-2 uninfected
AA-2/HIV-1
AA-2LSNnef/HIV-1
AA-2LSN-PAK140/HIV-1
AA-2TAR-PAK140/HIV-1
RT activity (cpm/ul)
5000
4000
3000
2000
1000
0
0
10
20
30
40
Days post infection

c.


Number of live cells
100
75
50
25
0
0
10
20
30
40
Days post infection

DAY SEVEN





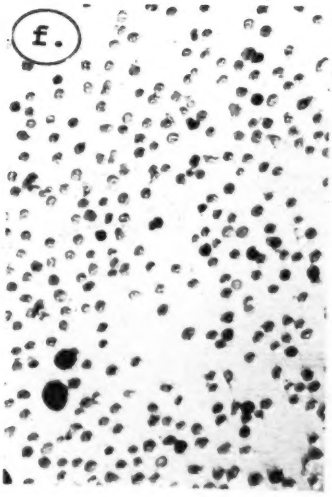
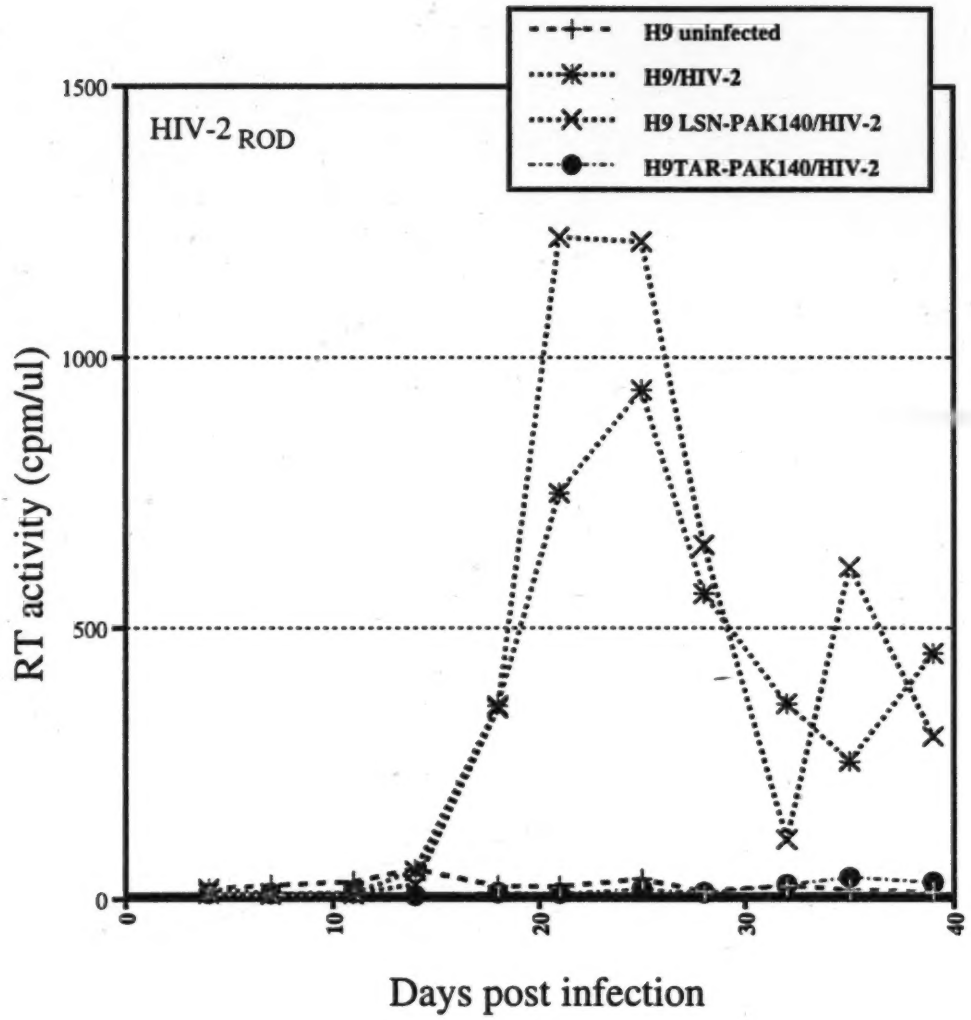


FIGURE 28. Kinetics of HIV virus production in wild-type and transduced H9 cells infected with HIV-2_{ROD}. H9 cells were transduced with the recombinant retroviruses LSN-nef, LSN-PAK140 or TAR-PAK140 and stable cell lines were established using G418 selection. Equal numbers of wild-type or transduced H9 cells were seeded into identical flasks (2×10^6 cells/25 cm^2/5 ml of media) and then were infected at time zero with of HIV-1_{ROD} at an MOI of 0.2 at time zero. Average RT activities for various culture supernatants are plotted versus time.


H9 uninfected
H9/HIV-2
H9 LSN-PAK140/HIV-2
H9TAR-PAK140/HIV-2
HIV-2 ROD
1500
1000
500
0
RT activity (cpm/ul)
0
10
20
30
40
Days post infection

observed when the TAR-PAK140 transduced AA-2 cells were challenged with HIV-2_{CBL} (Figure 29). (Note that in these experiments the particular cell line/strain combinations used were chosen simply for reasons of convenience).

4. TAR-PAK140 transduced H9 cells retained expression of CD4, whereas TAR-PAK140 transduced AA-2 cells showed reduced or even absent CD4 expression.

The diminished HIV virus production in the H9 TAR-PAK140 and AA-2 TAR-PAK140 cell lines could result if these transduced cell lines had lost expression of the CD4 surface marker, since they would no longer be susceptible to HIV infection. To test this possibility we examined the (uninfected) wild-type and transduced cell lines by CD4 staining and FACScan analysis. The FACS profiles for the H9 cells are shown in Figure 30, and for the AA-2 cells in Figure 31. In the case of H9 cells, levels of CD4 expression were similar between the control untransduced cells and all three of the stably transduced cell lines. In contrast, mean CD4 expression was reduced about five fold in the LSN-PAK140 transduced AA-2 cells (Figure 31c), and cells transduced with TAR-PAK140 showed a bi-modal staining profile, with either reduced or absent CD4 staining (Figure 31d).

5. Higher levels of MLV vector-derived transcripts were found in the uninfected TAR-PAK140 transduced cell lines compared to the LSN-PAK140 transduced cell lines.

Within the transduced H9 and AA-2 cell lines challenged with HIV, the 'packaging decoy' or antisense elements of PAK140 most likely exert their effect at the level of transcribed RNAs. Thus we examined the various cell lines for transcripts arising from the transduced recombinant proviruses (using a MLV specific probe), and these results are shown in Figure 32. Although transcripts were present in all the transduced cell lines, the

FIGURE 29. Kinetics of HIV virus production in wild-type and transduced AA-2 cells infected with HIV-2_{CBL}. Wild-type and transduced AA-2 cells were infected with HIV-2_{CBL} at an MOI of 0.002.

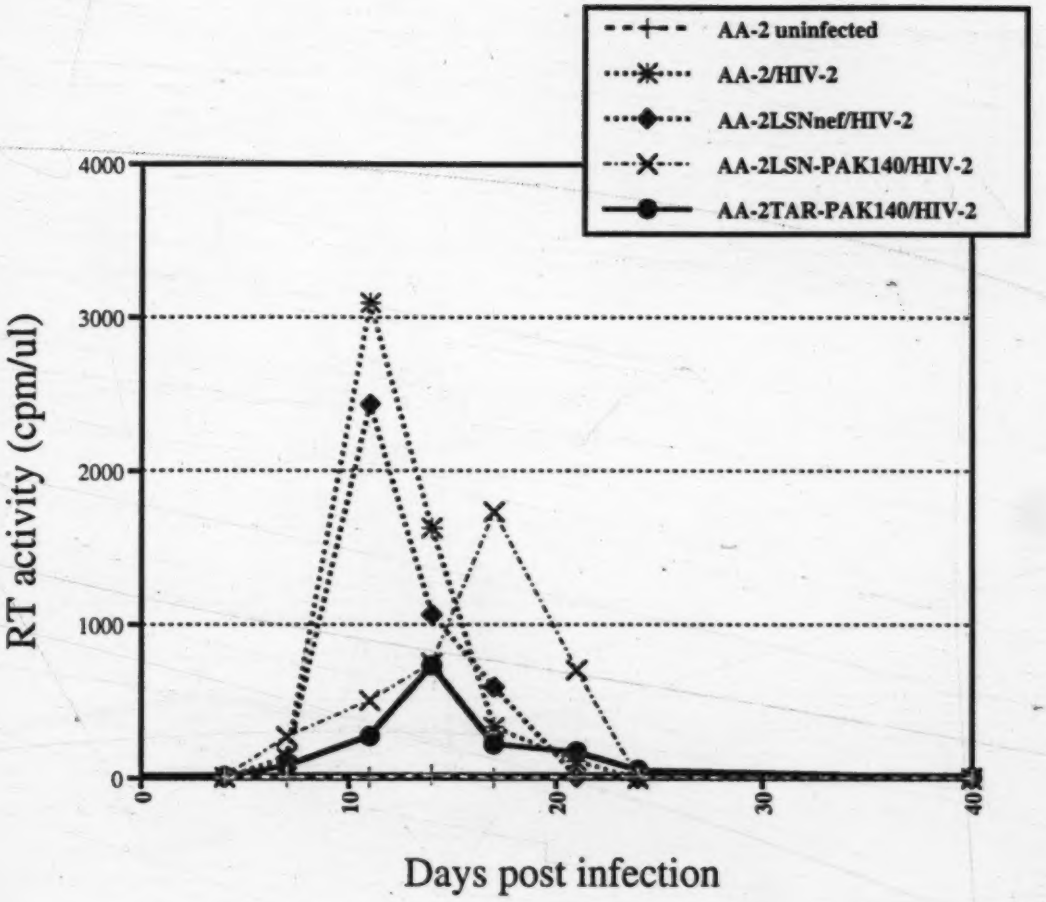

AA-2 uninfected
AA-2/HIV-2
AA-2LSNnef/HIV-2
AA-2LSN-PAK140/HIV-2
AA-2TAR-PAK140/HIV-2
RT activity (cpm/ul)
4000
3000
2000
1000
0
0
10
20
30
40
Days post infection

FIGURE 30. FACScan analysis of wild-type and transduced H9 cells stained with anti-human CD4 antibody. (**a**) wild-type H9 (**b**) LSN-nef transduced H9 (**c**) LSN-PAK140 transduced H9 (**d**) TAR-PAK140 transduced H9. The profile obtained from wild-type H9 cells stained with an isotype control is superimposed (black, filled).

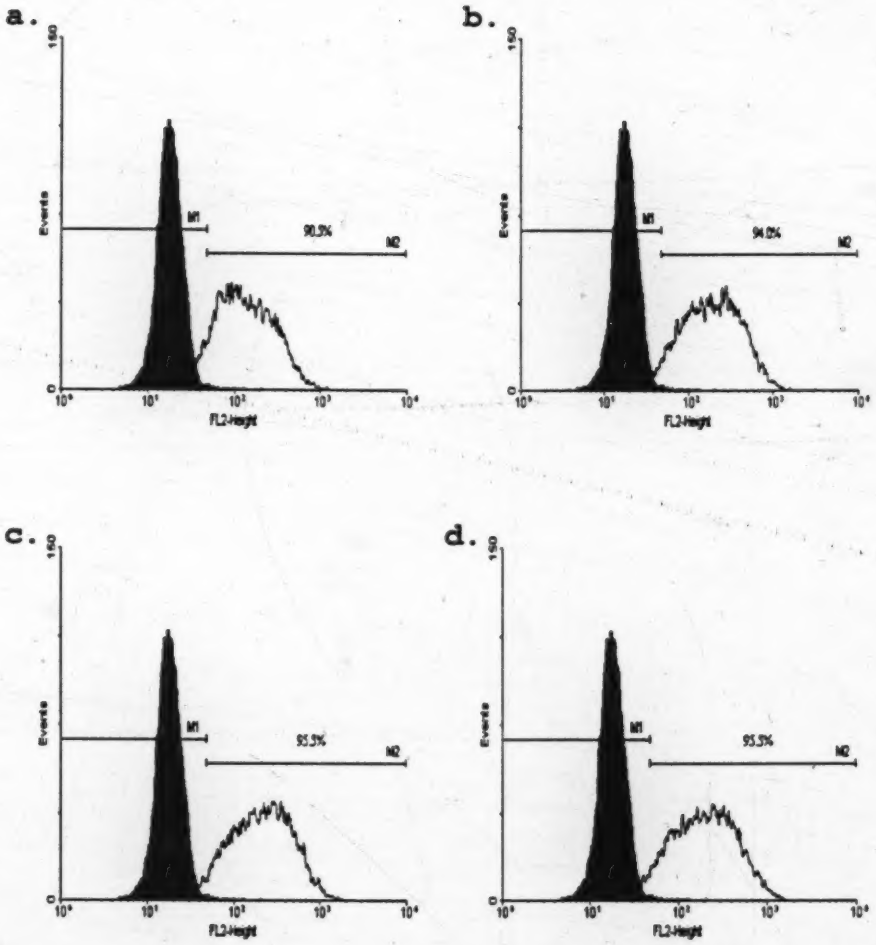
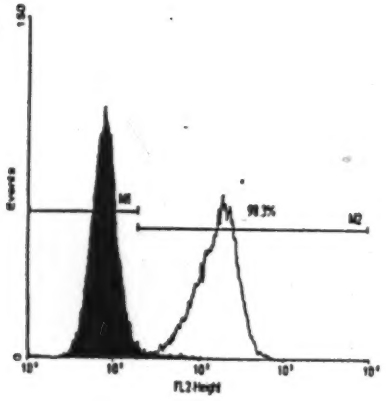
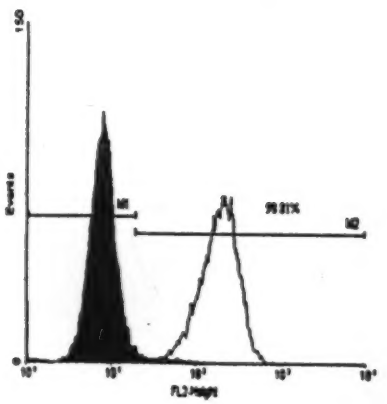

a.
b.
c.
d.
Events
FL2-Height
M1
M2
90.5%
94.0%
95.5%
95.5%

FIGURE 31. FACScan analysis of wild-type and transduced AA-2 cells stained with anti-human CD4 antibody. (**a**) wild-type H9 (**b**) LSN-nef transduced AA-2 (**c**) LSN-PAK140 transduced AA-2 (**d**) TAR-PAK140 transduced AA-2. The profile obtained from wild-type H9 cells stained with an isotype control is superimposed (black, filled).

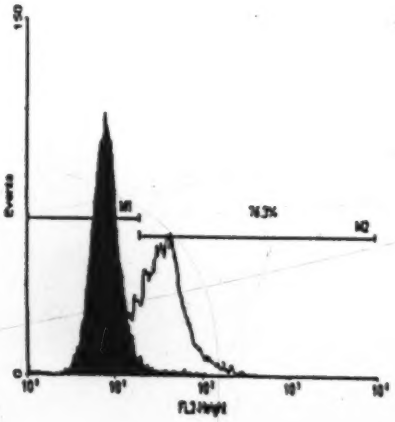
a.


150
Events
0
M1
98.3%
M2
FL2-Height

b.


150
Events
0
M1
99.81%
M2
FL2-Height

c.


150
Events
0
M1
76.3%
M2
FL2-Height

d.

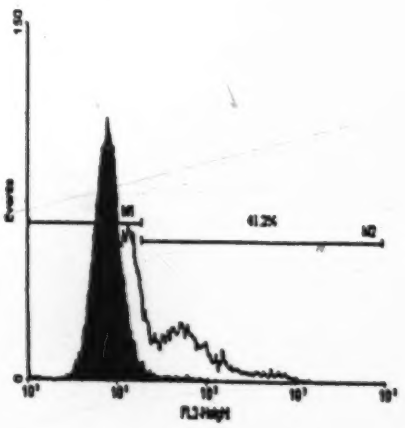
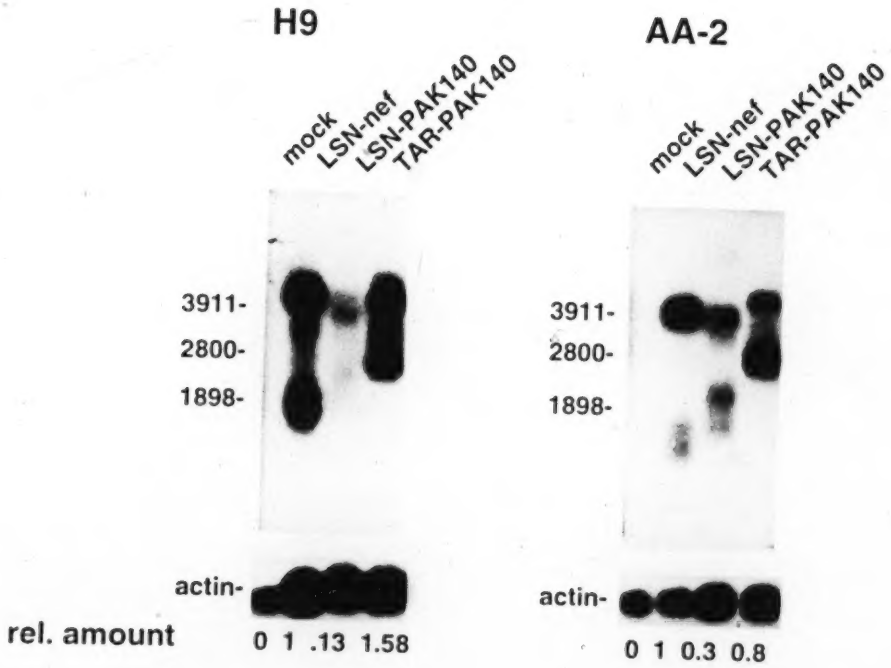

150
Events
0
M1
41.2%
M2
FL2-Height

FIGURE 32. Autoradiograph of a Northern blot examining retroviral transcripts present in H9 and AA-2 cell lines transduced with LSN-nef, LSN-PAK140, or TAR-PAK140. Poly A+ RNA was separated on a 1% formaldehyde-agarose gel, transferred to nitrocellulose and probed with the 1394 Bgl II-Sal I fragment from pLSN-PAK140 (Figure 18). This fragment contains Neo and PAK140 sequences. Using a Fuji BAS 1000 phosphoimager the integrated viral RNA signal (from all bands) was compared to the actin signal and this ratio is listed below the actin blot.


H9
AA-2
mock
LSN-nef
LSN-PAK140
TAR-PAK140
mock
LSN-nef
LSN-PAK140
TAR-PAK140
3911-
2800-
1898-
3911-
2800-
1898-
actin-
actin-
rel. amount
0 1 .13 1.58
0 1 0.3 0.8

H9 cells with TAR-PAK140 produced about 12 fold more MLV-derived RNAs than did the LSN-PAK140 transductants. The same general trend was seen in the AA-2 transduced cell lines, although in this case the TAR-PAK140 cells contained only about 3-fold more MLV-derived RNA than did the LSN-PAK140 cells. The control cells transduced with LSN-nef showed surprisingly high levels of MLV derived transcripts, roughly comparable to those present in TAR-PAK140. The size of the bands on these autoradiographs suggest transcripts of about 4500 bases for TAR-PAK140 and 3900 for the LSN vectors, which are in keeping with the size of the transcripts predicted from the original DNA constructs. In addition there is a 2800 base transcript in the TAR-PAK140 cells which would also be expected from the slice donor and acceptor sites present in the MLV portion of the vector (6). Note that this spliced transcript still contains the PAK140 sequence elements. LSN-nef shows an even smaller transcript which is probably due to cryptic splice sites present in the vector.

6. When cell lines chronically infected with tat-positive HIV-1_{NL4-3} were secondarily transduced with TAR-PAK140, production of infectious virus was reduced; this effect was not seen in the equivalent cell line infected with a tat-minus NL4-3 variant.

Because chronically infected cell lines were readily available, and because they perhaps provide a better model for gene therapy in HIV infected individuals, we transduced several of these chronic lines with LSN-PAK140, with TAR-PAK140, or with a control virus, TAR-CAT, and then measured HIV virus production (as infectious units) over a defined period of time (12 h). The results for several repeat experiments are summarized in Table 6. In addition to lines chronically infected with wild-type (i.e. Tat+) HIV_{NL4-3}, we also tested the equivalent cell line chronically infected with a tat minus version of HIV_{NL4-3}. The

TABLE 6. Infectious virus produced over 12 hours from cell lines chronically infected with HIV-1 and then secondarily transduced with LSN-PAK140, TAR-PAK140 or TAR-CAT.

Original cell line	No transduced virus	Transduced with		
		LSN-PAK140	TAR-PAK140	TAR-CAT
Molt.tat+$_{NL4-3}$				
experiment 1	2190 (574 cpm/ul)	ND	1275 (716 cpm/ul)	ND
experiment 2	4685 (1312 cpm/ul)		965 (582 cpm/ul)	
MT4.tat+$_{NL4-3}$				
experiment 1	885 (320 cpm/ul)	1090 (856 cpm/ul)	110 (360 cpm/ul)	515 (133 cpm/ul)
experiment 2	470 (172 cpm/ul)	635 (547 cpm/ul)	45 (314 cpm/ul)	580 (140 cpm/ul)
experiment 3	385 (153 cpm/ul)	435 (325 cpm/ul)	40 (166 cpm/ul)	450 (87 cpm/ul)
MT4.tat-$_{NL4-3}$				
experiment 1	610 (433 cpm/ul)	975 (456 cpm/ul)	630 (321 cpm/ul)	ND
experiment 2	1205 (362 cpm/ul)	860 (401 cpm/ul)	1150 (204 cpm/ul)	
experiment 3	935 (291 cpm/ul)	750 (451 cpm/ul)	935 (132 cpm/ul)	

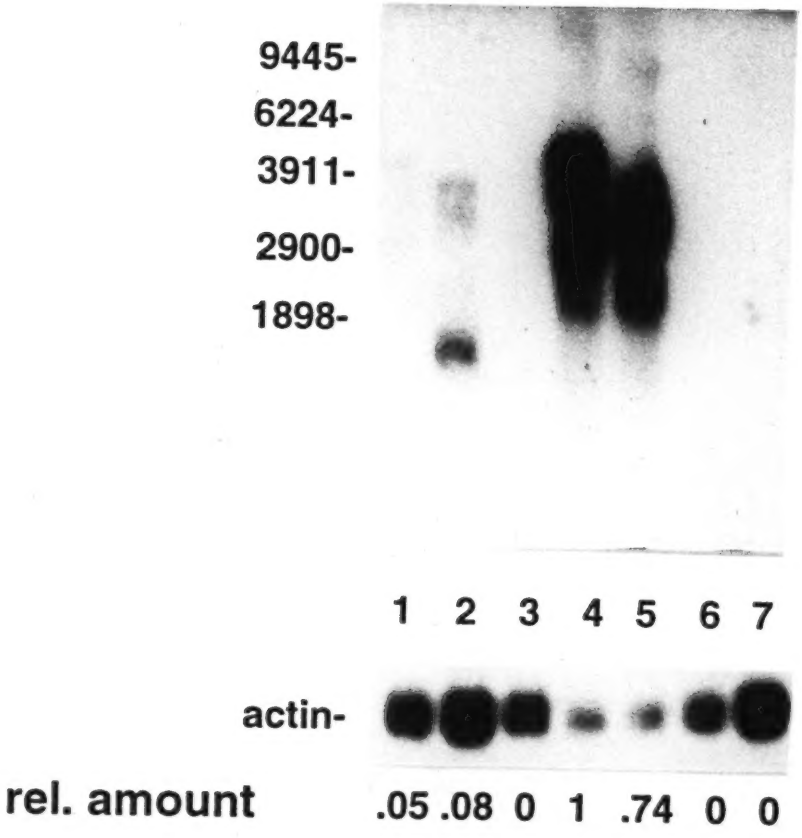
Cells that had survived HIV-1$_{NL4-3}$ infection (or infection with a Tat minus variant of HIV-1$_{NL4-3}$) were used to establish lines (Molt.tat+$_{NL4-3}$, MT4.tat+$_{NL4-3}$, and MT4.tat-$_{NL4-3}$) which chronically shed HIV virus. These were then transduced with the various recombinant retroviruses and selected with G418. The resulting cell lines were plated in six well plates (2 x 10^5 cells /0.5 ml/well) and culture supernatants harvested 12 h later. The supernatants were titered by infecting monolayers of HeLa CD4+ cells, and after 72 h staining with human immune serum and counting infected foci. These foci are expressed as IU/ ml. In addition to enumerating infectious HIV viruses, an aliquot of the supernatant was assayed for RT activity (shown in brackets). TAR-CAT is a control virus which contains the chloramphenicol acetyl transferase gene in place of PAK140.

use of the tat minus infected line allowed us to assess the effect (if any) of the Tat protein on the anti-viral effect of TAR-PAK140. The major result seen in Table 6 is that when TAR-PAK140 was transduced into two different cell lines chronically infected with a tat+HIV virus, there was a reduction in the amount of virus produced (as measured in infectious units). In general, this reduction was not seen when the same chronically infected cell lines were transduced with LSN-PAK140 or with TAR-CAT. Interestingly, the levels of infectious virus units were not necessarily mirrored in the RT activity of the supernatants (shown in brackets in Table 6), this point will be discussed further below. This last point and in particular the differences between this culture system and those shown previously will be dealt with further in the discussion.

7. Chronically infected MT4.tat+$_{NL4-3}$ cells transduced with the LSN-PAK140 or TAR-PAK140 both showed high levels of MLV vector-derived transcripts, but these transcripts were nearly absent in the MT4.tat-$_{NL4-3}$ cells.

To compare levels of MLV vector-derived transcripts in the cell lines chronically infected with tat plus or tat minus HIV and secondarily transduced with LSN-PAK140 or TAR-PAK140 we preformed Northern blot analysis. The results from a representative experiment are shown in Figure 33. In the MT4.tat-$_{NL4-3}$ cells transduced with LSN-PAK140 there were minimal transcripts produced (lane 2), and there were barely detectable transcripts in the comparable tat-, TAR-PAK140 transduced cells (lane 1). In contrast, in the MT4.tat+$_{NL4-3}$ cells there were very high levels of vector-derived transcripts in both the LSN-PAK140 and TAR-PAK140 transduced cell lines. As in Figure 32, the transcript sizes are entirely consistent with those expected from the LSN-PAK140 and TAR-PAK140 constructs.

FIGURE 33. Northern analysis of retroviral RNAs in HIV infected MT4.tat+$_{NL4-3}$ and MT4.tat-$_{NL4-3}$ cell lines transduced with the recombinant retroviruses LSN-PAK140 or TAR-PAK140. These cell lines were established using G418 selection as described in Table 6, and Poly A+ RNAs analyzed using the same probe as for Figure 30. Lane 1, MT4.tat-$_{NL4-3}$ transduced with TAR-PAK140; Lane 2, MT4.tat-$_{NL4-3}$ transduced with LSN-PAK140; Lane 3, MT4.tat-$_{NL4-3}$; Lane 4, MT4.tat+$_{NL4-3}$ transduced with TAR-PAK140; Lane 5, MT4.tat+$_{NL4-3}$ transduced with LSN-PAK140; Lane 6, MT4.tat-+$_{NL4-3}$; Lane 7, MT4 (i.e. uninfected parental cells). RNA molecular weight markers are shown to the left. Note that the actin signal in lanes 4 and 5 is relatively weaker, and this was done deliberately in order to compensate for the intensity of the MLV signal present in these lanes.


9445-
6224-
3911-
2900-
1898-
1 2 3 4 5 6 7
actin-
rel. amount
.05 .08 0 1 .74 0 0

Although somewhat surprising, the same experimental result (i.e. high levels of transcripts in both the LSN-PAK140 and TAR-PAK140 transduced cells) was obtained on three separate occasions.

8. H9 cells stably transduced with TAR-LJC140 did not give rise to reduced levels of virus following HIV-1 or HIV-2 infection.

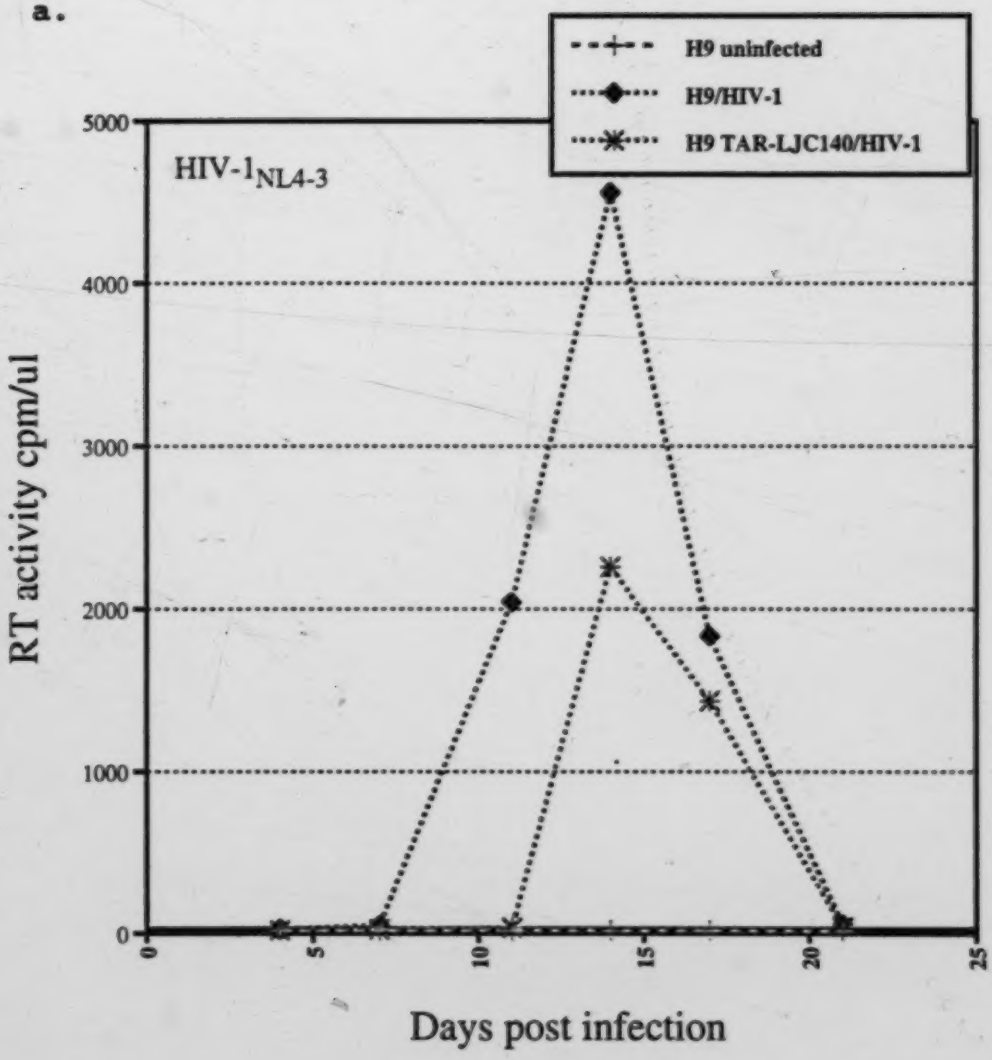
As explained in Section B above, two 'packaging decoy' constructs were made, PAK140 and LJC140. All of the in vitro experiments described above utilized PAK140. When PAK140 was discovered to have some anti-viral effect, this construct was carefully re-characterized and unexpectedly found to have 40 bases antisense to gag, rather than in the sense orientation. Once this fact was discovered, we went back to test LJC140 (which contains the packaging decoy all in the sense orientation) in similar in vitro assays as had been used for PAK140. Results of these experiments indicated that LJC140 transduced H9 cells were essentially identical to untransduced H9 cells in terms of their ability to support HIV-1 replication , using MOIs of 0.02 (Figure 34a) and 0.2 (Figure 34b). This also held for HIV-2 replication at an MOI of 0.02 (Figure 35).

9. Kinetics of HIV-1 virus production were similar in parental H9 cells and H9 cells that had been stably transduced with proviruses containing LJC140+gag antisense elements or PAK140+ a gag antisense element.

While a retroviral packaging decoy by itself may not have any anti-viral effect, it may still have a positive influence on a second antiviral element through co-localization (see Chapter 1). To test whether LJC140 or PAK140 could promote the anti-viral effects of larger elements antisense to gag, we constructed TAR-LJC140.antigag625, TAR-

FIGURE 34. Kinetics of HIV virus production in wild-type and transduced H9 cells infected with HIV_{NL4-3}. H9 cells were transduced with the recombinant retrovirus TAR-LJC140 and stable cell lines were established using G418 selection. Equal numbers of wild-type or transduced cells were seeded into identical flasks (2×10^6 cells/ $25cm^2$/ 5 ml of media) and then infected with $HIV\text{-}1_{NL4-3}$ at time zero. Aliquots of culture supernatant (2 ml) were harvested from each flask at 3-4 day intervals, and the volume removed was replaced with fresh media each time. Supernatants were stored at -80°C and an RT assay performed on all samples at the end of each study. RT assays were done in duplicate for each sample, and average values are shown; the duplicates typically gave values with 1% of each other. Average RT activities for various culture supernatants are plotted versus time, using an initial MOI of 0.02 (**a**) or 0.2 (**b**).

a.


H9 uninfected
H9/HIV-1
H9 TAR-LJC140/HIV-1
HIV-1NL4-3
RT activity cpm/ul
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
Days post infection

b.

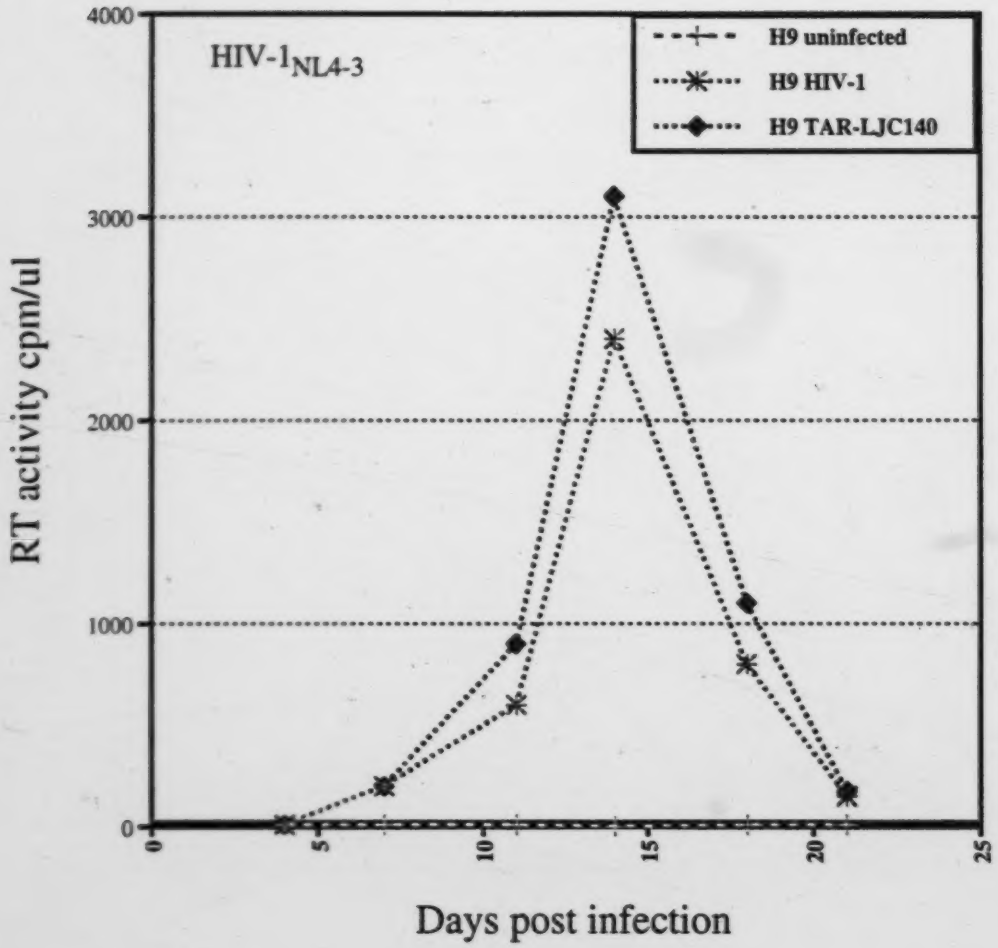
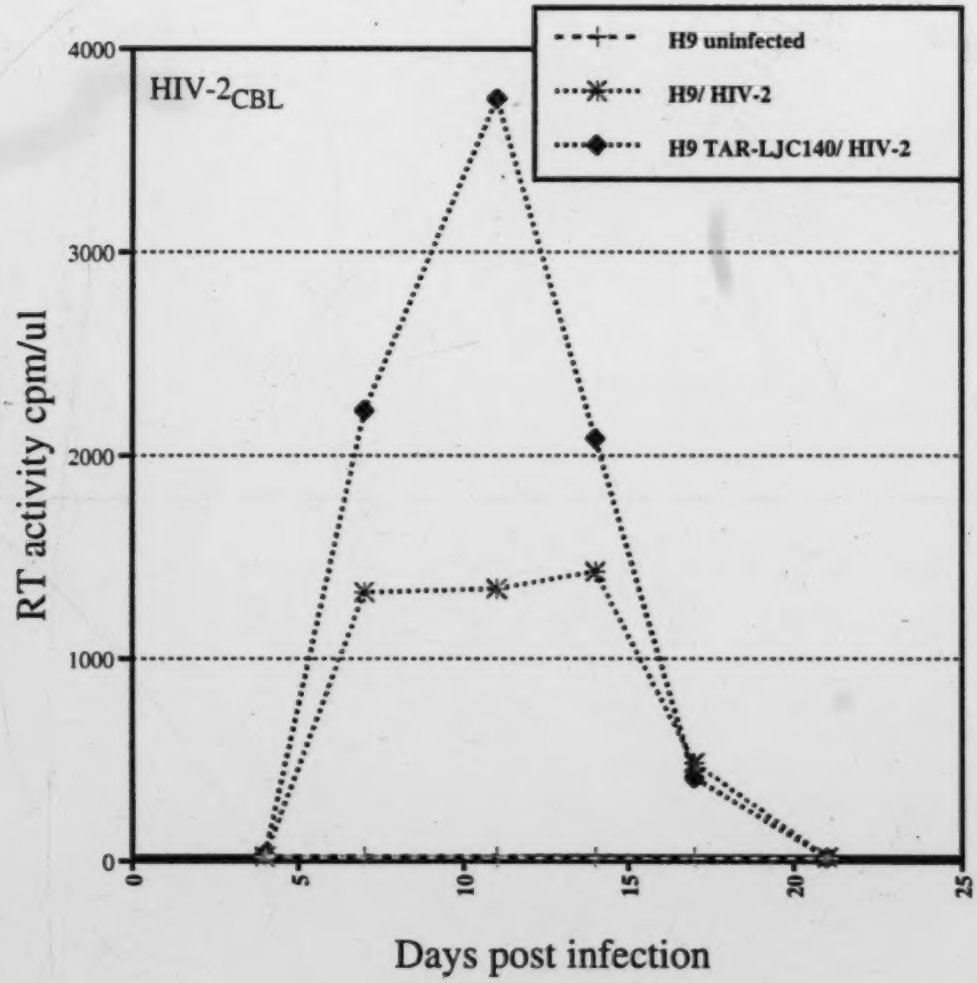

HIV-1NL4-3
H9 uninfected
H9 HIV-1
H9 TAR-LJC140
RT activity cpm/ul
4000
3000
2000
1000
0
0
5
10
15
20
25
Days post infection

FIGURE 35. Kinetics of HIV virus production in wild-type and TAR-LJC140 transduced H9 cells infected with HIV-2_{CBL}. This experiment is identical to that described in Figure 32a except that it was a different strain of HIV virus.

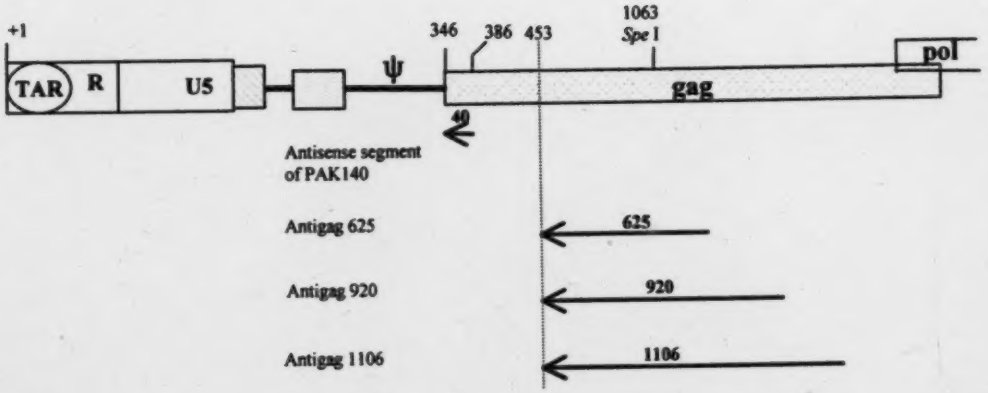

H9 uninfected
H9/ HIV-2
H9 TAR-LJC140/ HIV-2
HIV-2CBL
4000
3000
2000
1000
0
RT activity cpm/ul
0
5
10
15
20
25
Days post infection

LJC140.antigag920, TAR-LJC140.antigag1106, and TAR-PAK140.antigag1106. These constructs are shown in Figure 36. Recombinant viruses from each of these constructs were transduced into H9 cells and cell lines were established using G418 selection. The resulting cell lines as well as the parental H9 cells were infected with HIV-1_{1648} at an MOI of 0.02, and culture supernatants followed for RT activity over time. The results of this experiment are shown in Figure 37; virus production appeared to be the same in all of the cell lines.

FIGURE 36. **(a)** Schematic of HIV-1 RNA (5' portion) together with the approximate size and location of the target regions of hybridization for various gag antisense transcripts. **(b)** Diagrams of TAR-PAK140, TAR-LJC140 and derivative vectors that contain gag antisense elements.

a.

HIV-1 RNA (5' portion)



b.

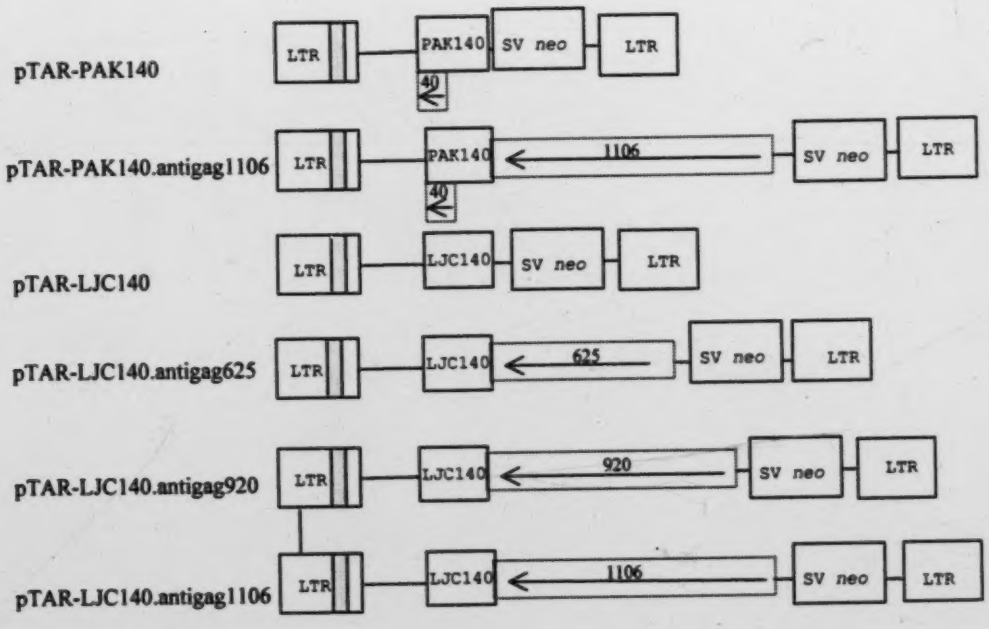
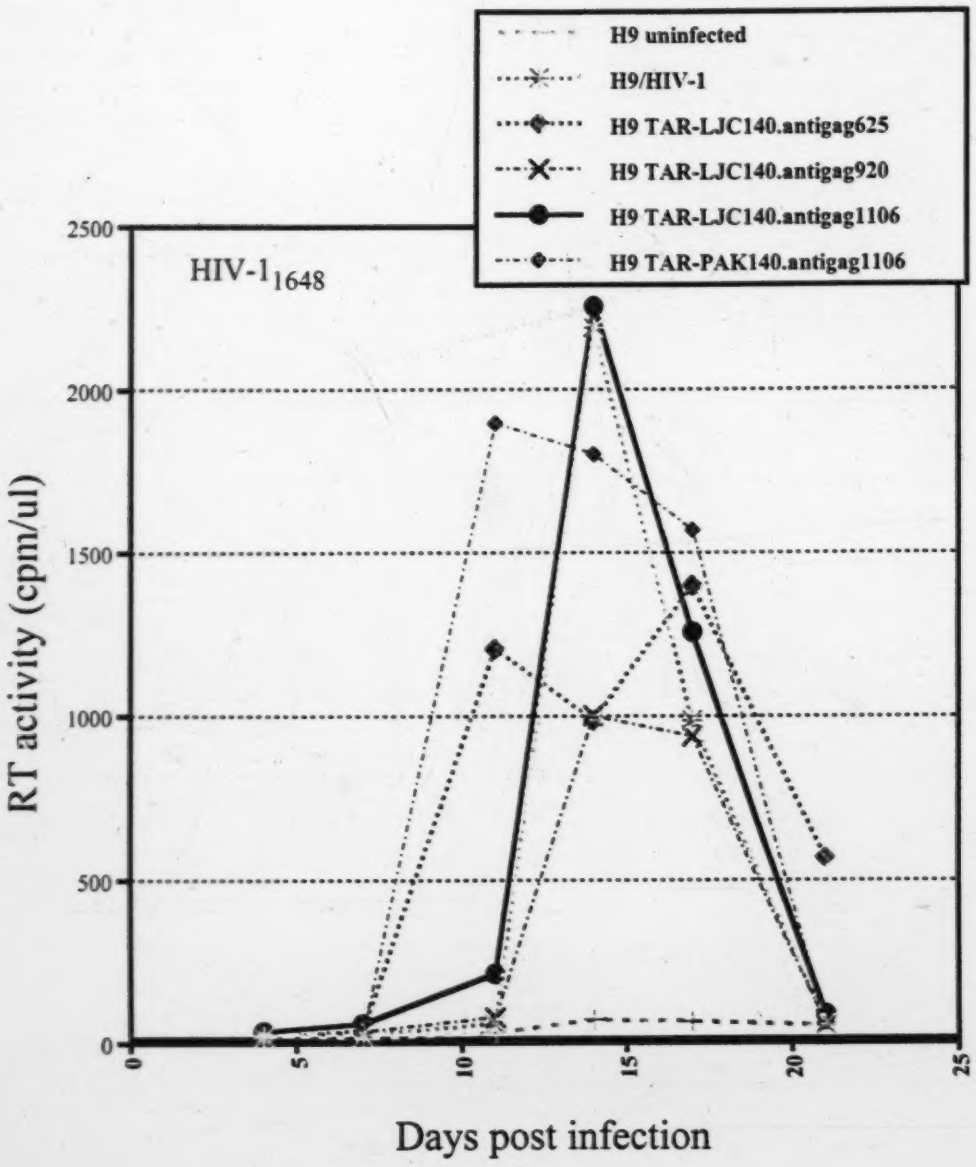


FIGURE 37. Kinetics of HIV virus production in wild-type and transduced H9 cells infected with HIV-1_{1648}. H9 cell lines were created that were transduced with one of the following viruses; TAR-LJC140.antigag625, TAR-LJC140.antigag920, TAR-LJC140.antigag1106, or TAR-PAK140.antigag1106. The experiment is similar to the previous kinetic experiments (e.g. Figure 25) except that the infecting strain is HIV-1_{1648} and the MOI used is 0.02. Note that HIV-1_{1648} behaves very similarly to HIV-1_{NL4-3} in tissue culture systems.


H9 uninfected
H9/HIV-1
H9 TAR-LJC140.antigag625
H9 TAR-LJC140.antigag920
H9 TAR-LJC140.antigag1106
H9 TAR-PAK140.antigag1106
HIV-1$_{1648}$
RT activity (cpm/ul)
2500
2000
1500
1000
500
0
0
5
10
15
20
25
Days post infection

D. Discussion

The initial antiviral constructs which we tested all contained antisense elements in the pLSN plasmid. These were co-transfected into HeLa cells along with pNL4-3, but none of them reduced the amount of virus released into culture supernatants, as determined indirectly using an RT assay (Figure 22). Since LSN is known to be a relatively weak promoter in HeLa cells it is likely that there were only low levels of antisense transcripts generated, and these were probably insufficient to have any anti-HIV effect. Alternately the antisense transcripts may have failed to hybridize with their target HIV sequences because of secondary structure constraints, or because they did not end up in the same compartments within the cell. These latter issues are difficult to predict based solely on primary sequence information; in fact at the present time the design of successful antisense elements is entirely empirical. It would have been more useful to test these same antisense elements in pTAR, which would have given higher levels of antisense transcripts, but this was not done because our focus shifted to packaging decoys, which gave more promising initial results.

It is challenging enough to design anti-HIV elements which could be used in the context of gene therapy, but it is perhaps even more difficult to find a relevant model in which to test such constructs. The HeLa cell co-transfection assay can be done easily and quickly (since no recombinant viruses are required), but it is also severely limited. A more relevant assay involves creating recombinant (potentially therapeutic) retroviruses, stably transducing these into susceptible cell types, and then challenging the transduced cells with HIV virus. This kind of assay has been labeled the 'acute infection assay'. In our acute infection assay we used two different human lymphoid cell lines, H9 and AA-2. The assay

involved adding infectious HIV virus at a relatively low multiplicity of infection (MOI), so that only a small proportion (e.g. 0.1-0.2%) of the cells are initially infected. These few cells produce viruses which in turn infect more of the cells, so that multiple rounds of virus production and infection ensue until finally all of the cells in the culture are infected. The cells used in these assays are susceptible to the cytopathic effects of HIV, and will die following infection (although occasional cells may survive to become chronically infected). Thus virus production peaks when the greatest number of cells have been infected but have not yet died. The amplification effect seen in the acute infection model means that a very minor effect occurring early in the infection can have a dramatic effect on subsequent virus production. For example, an agent which partially inhibits virus production in this model may delay the occurrence of the viral peak, and/or reduce its magnitude. The acute infection model is very different from the 'chronic infection model', where cells which are already infected with HIV virus are secondarily transduced with a potential therapeutic retrovirus, as described in more detail below.

The first packaging decoy construct which we tested was PAK140. This consisted of a sense strand packaging element together with a short gag anti-sense sequence, rather than the 'all-sense' strand packaging element contained in LJC140. H9 cells transduced with PAK140 in a Tat-responsive virus (TAR) were dramatically resistant to HIV-1 infection in an acute infection assay (no clear peak of virus in 28 days, MOI 0.001; or in 40 days, MOI 0.002; Figure 25). In contrast, the same PAK140 element in the LSN virus (contains a weaker promoter unresponsive to Tat) did not confer resistance. This apparent resistance to HIV-1 infection was further confirmed in the TAR-PAK140 transduced H9 celis by our

observation that they were not killed by the HIV virus, and very few cells contained viral antigen at 14 days post infection. The TAR-PAK140 transduced H9 cells also appear to be relatively resistant to a second, less virulent strain of HIV-1 used at a MOI of 0.2 (Figure 26). In this case a peak of virus was generated from the transduced H9 cells, but the peak was delayed and of a lower magnitude. This latter result suggests that rather than completely inhibiting HIV-1 production (as inferred by our original results), TAR-PAK140 transduction has a partial inhibitory effect. In this context, it may be that if we had followed the original H9 cultures for a longer period we might have eventually detected a viral peak. Clearly the pathogenicity of the virus and the MOI used both affect what is observed in the H9 acute infection model.

The mechanism by which TAR-PAK140 transduced H9 cells resist HIV-1 infection is unclear. None of the transduced cells lost CD4 expression either before or after HIV infection (Figure 30 and data not shown), thus discounting this as a possible mechanism of resistance. Northern blots indicated that the 'HIV-1 resistant' TAR-PAK140 transduced H9 cells had a much higher level of MLV-derived transcripts than did the LSN-PAK140 transduced (HIV susceptible) cells, suggesting that resistance is correlated with the presence of high levels of the PAK140 transcripts. In this context it is important to note that even the shorter (singly spliced) transcript from TAR-PAK140 would contain the PAK140 element (as well as the TAR element which might also be having an effect). There are several levels at which these transcripts may be acting to produce viral resistance. They may give rise to large amounts of dsRNA (either directly or by hybridizing with HIV RNA), and this may induce the interferon system within the infected cells thus protecting neighboring cells. The

transcripts may interfere with translation of HIV RNA, although the gag antisense segment of PAK140 may be too short to accomplish this by direct hybridization. The TAR element present in the transcripts may sequester HIV Tat protein and thus interfere with HIV viral replication. One of the most interesting possibilities is that because of the packaging decoy present in PAK140, one MLV transcript may be co-packaged with one HIV transcript to form a non-infectious 'heterozygous' viral particle. In theory such defective particles might also result when two MLV transcripts were packaged. Finally, a combination of several of these mechanisms may be acting together to achieve the anti-viral effect.

In the various H9 acute infection assays it is interesting that the LSN-nef transduced cells (meant to be included as a negative control) consistently showed a delay in peak virus production, although the magnitude of this peak was not significantly or consistently reduced. The LSN-nef transduced cells clearly remained CD4+, and the reason for the delay in the viral peak is probably due to the effects of Nef on HIV transcription. Indeed Nef stands for 'negative effector of transcription', and it has been shown by several groups to down-regulate HIV transcription (3) (although not everyone has obtained this result (147). The other point to be made about the LSN-nef transduced H9 cells is that (despite the relatively weak LSN promoter) they showed very high levels of MLV-derived transcripts (Figure 32). Why these transcript levels are so high in the nef transduced cells (even prior to HIV-1 infection) is unclear, but it may relate to a preferred integration site or to an effect of the nef protein itself. Whatever the reason for the high transcription levels, these transduced cells do contain abundant MLV derived transcripts (i.e. levels similar to those seen in TAR-PAK140), yet they are not as able to resist infection by HIV-1. Thus high

levels of MLV-derived transcripts *per se* do not account for the resistance to HIV seen in the TAR-PAK140 transduced H9 cells.

The results obtained with the transduced AA-2 cells were not as clear-cut as those for the H9 cells. In the acute infection assay, the TAR-PAK140 transduced AA-2 cells showed a clear peak in the RT activity, which was consistently delayed (by about 4 days) and of lower magnitude than the control cultures (20% in one experiment, 4% in another). Even where peak RT level was only 4% of controls, there was significant cell death in the AA-2 cultures compared to the corresponding H9 cultures (compare Figures 27c and 25c). However, at early time points (i.e. 7 days post-infection) there was less HIV-1 antigen present in the TAR-PAK140 transduced AA-2 cells compared to the control cells (Figure 27d-g; the HIV-1 infection was clearly delayed in the TAR-PAK140 AA-2 transductants). Overall these results are in keeping with the fact that AA-2 cells are much more sensitive to the cytopathic effects of HIV-1 than are H9 cells. The AA-2 results may also be consistent with the idea that transduction with the TAR-PAK140 confers partial resistance to HIV-1 and/or inhibits viral replication, but the issue is clouded by the fact that the TAR-PAK140 transduced AA-2 cells consisted of a large population of CD4 negative cells even prior to HIV infection (Figure 31). One simple explanation for the AA-2 results is that fewer cells were initially infected (the CD4 negative cells would be resistant), which meant that less total virus was made and the peak virus production was delayed slightly. Since the AA-2 results could be explained entirely by the loss of CD4 expression, they provide no clear information about the effects of TAR-PAK140 on HIV-1 replication. Just why the TAR-PAK140 transduced AA-2 cells (and to a lesser extent the LSN-PAK140 transduced AA-2 cells)

became CD4 negative is unclear, but these results do point out one of the pitfalls of the acute infection model which must be taken into account whenever testing any potential anti-HIV constructs. Interestingly, the LSN-nef transduced AA-2 cells also showed relatively high levels of MLV derived transcripts, in keeping with the results seen for the H9 LSN-nef-transduced cells.

Consistent with the HIV-1 results, when the TAR-PAK140 transduced H9 cells were infected with HIV-2 there was also no clear peak of RT activity in the first 40 days following infection. Although a viral peak may have been detected if these HIV-2 infected cultures had been followed for longer times, in comparison to the control cell lines the TAR-PAK140 transduced H9 were clearly resistant to HIV-2 infection. The mechanism for HIV-2 resistance is probably similar to that for HIV-1; leading possibilities include an interferon effect and the possibility of co-packaging heterozygous genomes giving rise to defective particles. Because of sequence differences between HIV-1 and HIV-2 the 40 base gag antisense element present in PAK140 would not be expected to hybridize with the HIV-2 genomic RNA. In the TAR-PAK140 transduced AA-2 cells infected with HIV-2 there was a reduced, but still significant peak of RT (25% of controls) which was delayed by a few days. Again these AA-2 results are similar to those obtained for HIV-1 on these cell lines. HIV-2 can use the CD4 receptor, but CD4 is not absolutely required for HIV-2 infection (111), so the absence of CD4 on the TAR-PAK140 transduced AA-2 cells may not have been as significant here as it was in the HIV-1 acute infection model.

The acute assay system provides us with information about the fate of cells transduced with potential therapeutic viruses and then infected with HIV. This type of assay

is only partially relevant to HIV infected individuals, since many of their cells will already contain the HIV virus. We might also envision that the therapeutic viruses could affect the fate of HIV infected cells. In keeping with these ideas, and because Dr. Chang had developed 'chronically' HIV infected cell lines which were readily available, we went on to test LSN-PAK140 and TAR-PAK140 in these cell lines. This provided a unique approach to assessing the anti-viral effects of our gene therapy vectors. Note that by 'chronically infected' we mean cells that have survived HIV infection and gone on to continuously shed virus.

Despite its attractions, the 'chronic infection model' is relatively complex as compared to the acute infection model. In the acute infection model (specifically using low MOIs) the establishment and amplification of an HIV infection is monitored in a population of uninfected cells over a 40 day period. If, for example PAK140 transduction caused the production of defective viral particles, this would have a dramatic effect on the timing and magnitude of peak virus production in the acute infection model. In contrast, chronically infected cells already have HIV stably integrated; they are continuously producing HIV virus. When such cells are transduced with a potentially therapeutic retrovirus and selected for G418 resistance, the cells that we eventually end up with are the result of a complex selection process. There are multiple factors that affect this selection and the final levels of HIV production that result. First, production of viral particles is a disadvantage to the cell, but transduced cells which produce a higher proportion of defective particles would not necessarily have a survival advantage over cells that produce the same quantity of infectious HIV particles (wild-type or defective particles both presumably exact the same physiological

toll). Second, continuous production of HIV-1 likely causes ongoing cytopathic effects (e.g. even MLV producer cell lines are unstable); cells transduced with a therapeutic virus that reduces these cytopathic effects will have an advantage and predominate in the culture. Although most or all 'MLV' transduction events will confer G418 resistance, those transduction events which at the same time reduce the cytopathic effects of HIV-1 production would tend to be selected for. Finally, for simple metabolic reasons, cells which are healthier will produce more HIV virus.

The other question which arises in either the acute or chronic assays of HIV infection is how best to monitor the quantity of virus produced. In fact, it is possible to titre HIV virus; this type of assay is accurate but time consuming and technically demanding. A much easier and faster assay involves simply measuring the RT activity in the culture supernatants. It is important to note that 'heterozygous' viral particles would still contribute RT activity, but would not be infectious. In chronic cultures a higher ratio of RT to infectious units suggests the presence of more defective particles (e.g. 'heterozygous' or even 'homozygous' for MLV).

In contrast to the acute infection assay where virus production was monitored for many weeks, in the assays of the chronically infected/transduced cells it was more appropriate to monitor virus production from equivalent numbers of freshly plated ('metabolically equivalent') cells over a relatively brief period of time. In the TAR-PAK140 transduced MT4.tat+$_{NL4-3}$ cells there were about 10-fold less infectious particles produced in 12 hours than were produced from the LSN-PAK140 transduced or untransduced cells. However, the RT levels present in the supernatants were not correspondingly decreased

(Table 6). Table 7 shows the data from Table 6 expressed as a ratio of RT activity per infectious unit; this ratio is much higher for the TAR-PAK140 transduced MT4.tat+$_{NL4-3}$ cells than for any other cell lines assayed. One explanation for this result is that these cultures were producing a higher portion of defective particles, possibly in the form of 'heterozygous' viruses.

In the MT4.tat-$_{NL4-3}$ (tat minus) cell lines, there were roughly the same number of infectious HIV particles produced from the untransduced and transduced cell lines. Northern blot results indicate that TAR-PAK140 RNA levels were very low in these cells, and although sufficient to confer G418 resistance, these were probably too low to have any other effect on the cells. The lower levels of transcripts in the absence of Tat are consistent with the results seen in Chapter 2. Since the tat minus virus is highly defective, it is unlikely to have caused as much cytopathic effect in the chronically infected lines, and transduction events which were selected for because they reduced the cytopathic effects in the tat plus infected lines would not necessarily be selected for in the 'tat minus' infected lines.

Surprisingly there were abundant MLV derived transcripts in both the LSN-PAK140 and the TAR-PAK140 transduced MT4.tat+$_{NL4-3}$ cell lines (Figure 33). This is understandable for the case of TAR-PAK140 since the promoter is Tat responsive, but the reason high levels of RNA occur in the LSN-PAK140 transduced cells is much less clear. These results, which were obtained on three separate occasions, appear contrary to the results in Chapter 2, or even in Figure 32 (H9 cells especially). One way out of this conundrum is to argue that the transduced, tat plus chronically infected cells represent a highly selected population. It would appear that for some reason the LSN-PAK140 transduced lines were

selected for cells which expressed high levels of the MLV transcripts. Integration events which occurred near a powerful endogenous promoter could lead to high RNA levels, since other studies have clearly shown that retroviral integration site can influence transcription rates. Although other factors that influence RNA stability will contribute to amounts of RNA. In fact it was noted that following transduction with TAR-PAK140 the MT4.tat+$_{NL4-3}$ cells readily grew to large numbers under G418 selection, whereas following LSN-PAK140 transduction it took much longer to generate equivalent numbers of cells (data not shown).

Why would cells expressing high levels of MLV-derived transcripts be selected for in the LSN-PAK140 transduced MT4.tat+$_{NL4-3}$ cell lines? There is if anything a little more infectious HIV virus and RT being produced from these cells compared to the parental chronically infected cells, which means there is no advantage conferred vis-a-vi levels of virus produced (although it may indicate that the cells are healthier. Perhaps the higher levels of LSN-PAK140 transcripts may somehow reduce the cytopathic effect of HIV on the chronically infected cells, through an unknown mechanism (it would not be through sequestering Tat, since there is no TAR in the LSN transcripts).

The high levels of transcripts present in the LSN-PAK140 transduced MT4.tat+$_{NL4-3}$ cell lines also raises another interesting question. Given the abundance of PAK140 transcripts in these cells, why are there not more defective particles made, as we have suggested for the TAR-PAK140 transduced MT4.tat+$_{NL4-3}$ cell lines? It may be that the LSN-PAK140 transduced cells do make some defective particles, since the ratio of RT to infectious units is slightly higher here than in any of the other control cell lines (Table 7). At the same time there is not a dramatic increase in RT per infectious unit which is seen in

the TAR-PAK140 transduced MT4.tat+$_{NL4-3}$ cell lines. It may be that in addition to the PAK-140 element itself, the presence of TAR (plus or minus attached Tat protein) is critical for packaging of the MLV-derived transcripts into defective particles. Although all of the above arguments are logically consistent, they are also highly speculative; clearly many more experiments would be required to characterize and dissect the chronically infected/transduced cell lines before a clear view of the mechanism could be obtained.

The final set of experiments in this chapter examined the effect of the LJC140 packaging decoy element on HIV-1 production. In fact, prior transduction of TAR-LJC140 into H9 cells did not inhibit HIV-1$_{NL4-3}$ production in the acute infection assay, a result which is clearly different to that which we obtained with TAR-PAK140. This may point to the importance of the element which is different between PAK140 and LJC140, namely the 40 base segment which was either antisense or sense to gag, although a much more trivial explanation for the different results can also be envisioned (see below). The short gag antisense element may promote dimerization of heterozygous genomes; clearly if it did hybridize to HIV-1 RNA it would bring the natural and synthetic dimerization domains close together (Figure 23c; the dimerization domain is contained in element A). However, such hybridization would not occur with HIV-2, which was also inhibited in the TAR-PAK140 transduced H9 cells, so this is unlikely to be the whole story. It would be interesting to determine if the 40 base antisense segment alone would have any effect in a TAR containing vector.

A more trivial explanation for the different results obtained for the TAR-PAK140 and TAR-LJC140 transduced H9 cell lines relates to the fact that different MOI's (HIV-1$_{NL4-3}$

TABLE 7. Ratio of RT activity per infectious unit* in the supernatants from cell lines chronically infected with HIV-1 and then secondarily transduced with LSN-PAK140, TAR-PAK140 or TAR-CAT.

Original cell line	No transduced virus	Transduced with		
		LSN-PAK140	TAR-PAK140	TAR-CAT
Molt.tat+$_{NL4-3}$				
experiment 1	0.26	ND	0.56	ND
experiment 2	0.28		0.60	
MT4.tat+$_{NL4-3}$				
experiment 1	0.36	0.78	3.3	0.26
experiment 2	0.37	0.86	7.0	0.24
experiment 3	0.40	0.75	4.15	0.19
MT4.tat-$_{NL4-3}$				
experiment 1	0.71	0.47	0.51	ND
experiment 2	0.30	0.47	0.18	
experiment 3	0.31	0.60	0.14	

Data from Table 6 was used to calculate a ratio of RT activity per unit of infectious HIV virus.

viruses per H9 cell) were used in the different experiments; the TAR-PAK140 experiments used MOI's of 0.001 or 0.002 (Figure 25 and onwards; excepting Figure 26 where the less pathogenic HIV-1_{ELI} was used at an MOI of 0.2), whereas the TAR-LJC140 experiments used MOI's of 0.02 or 0.2 (Figure 34). Although these latter MOI's are as high or higher than have been used in these types of studies, Dr. Chang felt that they were more appropriate in this series of experiments. Unfortunately no side-by-side comparison was made between the TAR-PAK140 and the TAR-LJC140 transduced H9 cells in the acute infection assay, the TAR-LJC140 experiments were done in a blinded fashion in collaboration with Dr. Chang, and unfortunately because of mis-communication the side-by-side experiment was not included. Time constraints did not allow the experiment to be repeated.

Our final TAR-LJC140 experiments made use of vectors which combined the LJC140 packaging decoy element (or the PAK140 element) with longer gag antisense elements. When transduced into H9 cells, none of these constructs was able to inhibit HIV-1_{1648} production in the acute infection model, using an initial MOI of 0.02. Motivation for these experiments arose from the fact that Veres et al. had reported some success with very long gag antisense constructs. However, there are significant differences between Veres work and our own experiments(353). These investigators selected for a single transduced clone that expressed very high levels of the antisense transcript, whereas heterogeneous transduced cell populations were used in all of our studies. Veres also used an MOI of 0.002, whereas we used 0.02 (353).

This last series of experiments did include one side-by-side comparison of the LJC140 and PAK140 elements, in the form of the TAR-LJC140.antigag1106 and TAR-

PAK140.antigag1106 transduced H9 cell lines. The TAR-PAK140.antigag1106 transduced H9 cell lines were clearly unable to inhibit HIV-1_{1648} production when infected at an initial MOI of 0.02. Why were these results so different from those which we had originally obtained using the TAR-PAK140 transduced H9 cells? Different MOIs and different HIV-1 viruses were used in these later experiments. In addition, it is possible that placing the antigag1109 element next to the PAK140 element alters the overall structure and function of the packaging and/or dimerization domains.

CHAPTER 4-FINAL DISCUSSION AND CONCLUSIONS

The science of gene therapy is a relatively new and exciting field. As it is still early days there exist many problems to be solved. One lesson to come from early clinical gene therapy trials is that the existing techniques are inadequate (12). Gene therapists have encountered difficulties in such fundamental areas, such as vector development, as gene transfer and gene expression. In 1995 Harold Varmus, director of the U.S. National Institute of Health (Bethesda Maryland) expressed concern that too much emphasis was being placed on the initiation of clinical trials. He felt that a return to basic research in the areas of gene therapy vectors and the cell biology of the target tissues was needed (225). Approximately 75% of all gene therapy clinical trials in the United States use a retroviral-based vector as the gene delivery system (225). The overall objective of this thesis was to design a better retroviral gene therapy system for anti-HIV applications.

Anti-HIV gene therapy is challenging, because the anti-viral gene must behave dominantly to suppress viral replication. For many of the therapeutic strategies, including delivering elements that create antisense or decoy RNAs, gene products have to be present in high concentrations to be effective in countering HIV replication. HIV transcribes it genome at very high rates so that anti-HIV genes would also have to be expressed at high levels. With this in mind our main goal in creating a retroviral vector for anti-HIV applications was to increase transcription rates of inserted genes, and to make the vector transcriptionally responsive to HIV. To accomplish this we added a CMV enhancer/promoter element and the HIV-1 TAR sequence to the LTR of an existing MLV

based vector. The resulting vector we named pLGCTSN in Chapter 2(and for brevity renamed pTAR in Chapter 3). We showed that pLGCTSN upregulated transcription from its LTR in the presence of Tat.

The properties of pTAR made it a good candidate for testing anti-HIV genetic elements. An anti-HIV element (PAK140) that was expected to behave as a 'packaging decoy' was cloned into pTAR. H9 cells stably transduced with recombinant viruses produced from the construct pTAR-PAK140 were challenged with HIV-1 and HIV-2. The presence of the TAR-PAK140 derived transcripts in H9 cells appeared to diminish HIV production from these cells.

Although we speculate that the effect of PAK140 in the H9 cells was to attenuate HIV infection H9 cells via the creation of defective particles this thesis does not provide any evidence to support this conclusion. There is an example in the literature of another group that tested a 'packaging decoy' element (31). Berkout and Wamel created a construct containing the wild-type HIV-1 LTR and the HIV-1 ψ element. When this construct was transfected into HeLa cells together with a HIV proviral plasmid the investigators observed reduced RT levels and a corresponding with reduction in protein synthesis (31). Our experiments with the TAR-PAK140-transduced HIV-1 chronic culture differ from Berkout and Wamels, since in our case the reverse transcriptase levels did not show an appreciable decrease as compared to the untransduced HIV-1 chronic cultures.

It would be interesting to explore the possibility that TAR-PAK140 derived RNA can be co-packaged with HIV genomes into viral particles. Experimentally such a demonstration might be possible using density gradient centrifugation. Virus could be harvested from the

TAR-PAK140 transduced MT4.tat+$_{NL4-3}$ cultures (chonically infected) and applied to a density gradient. If there were viruses present with different genomic RNAs they could have slightly different densities. Particles that contained the vector based sequences either in one copy or two should be lighter than particles that contained wild-type HIV derived genomes since the vector transcript is shorter than the HIV genome. Once the particles were isolated on an appropriate gradient it would be possible to confirm the presence of the various RNA's by RT- PCR analysis or even hybridization. Alternatively one could use an approach based on the hybridization of oligonucleotides to viral encapsulated genomes. Oligonucleotides with sequences specific for MLV derived transcripts or for the HIV-1 genome could be tagged with different colored fluorescent labels and hybridized to permeablized viral particles isolated from TAR-PAK140 transduced MT4.tat+$_{NL4-3}$ cultures. After hybridization one could examine the viral particles by confocal microscopy, in this scenario a particle with a 'heterozygous genome would emit at both wavelengths and give a unique final colour. If it could be shown that the HIV based particle did indeed contain vector derived sequence it would help define the mechanism of action of PAK-140.

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APPENDIX A-CONSTRUCTION OF THY CONTAINING VECTORS

A. Vector modification designed to increase long term stability of expression

Several mechanisms have been implicated in the transcriptional silencing of the MLV LTR, it is likely that they also function to some degree in silencing the modified LTR's. One of these, de novo methylation of cytosine residues has been detected in embryonic cell lines (57). The methylation of cytosine residues results in a irreversible block to transcription initiation. For retroviral mediated gene transfer into haematopoietic stem cells a lack of expression was observed that was presumed to be due to the same repression mechanisms observed in cell lines (59). It has been shown that loss of expression occurs without apparent loss of sequence, and that the frequency of inactivation is dependent on the chromosomal position of the vector sequences (57).

Since bone marrow stem cells are an important target for human gene therapy it is a concern of this thesis to improve the long-term expression of the retroviral vectors discussed in the previous chapter. We attempted to produce a retroviral vector that would overcome the inherent inactivity of the MLV transcriptional unit in embryonic cells. To increase the stability of expression from the MLV derived LTRs a demethylation fragment from the 5' upstream region of the murine Thy-1 gene was introduced into the 3' LTR of pLSNcat and pLGCTSNcat. This sequence has been reported to inhibit de novo methylation of adjacent heterologous sequences in a plasmid transfected into embryonic stem cells (338). DNA is extensively methylated de novo when introduced into pluripotent cells. The CpG islands of the mouse Thy-1 gene does not become methylated when introduced into embryonic mouse

stem cells. A 214-base-pair region near the promoter of the Thy-1 gene protects itself as well as surrounding DNA sequences (338). This sequence has been reported to inhibit de novo methylation of adjacent heterologous sequences in a plasmid transfected into embryonic stem cells (338). Other groups have shown that the shut-down of expression in tissue culture cells is due to methylation (158).

B. Methods

1. Cloning of pLSNthycat and pLGCSTNthycat

The plasmid pKSthy was a gift of M Syzff, it contained the Thy-1 demethylation sequence-bounded by the restriction sites *Sma*I and *Xba*I. To facilitate future cloning the original vector was cut with the enzyme *Sma*I and a linker containing the site for *Nhe*I was cloned into the open vector, this resulted in the clone pthyNhe. The ultimate aim of this cloning exercise is to add the Thy-1 sequence to the gene therapy vectors pLSN and pLGCTSN at the 3' LTR. Details of the cloning of pLGCTSN.Thy-cat are shown in Figures 38-40, pLSN.Thy.cat is cloned using a similar strategy.

These vectors (pLSN and pLGTSN) contain two LTRs with nearly identical restriction sites, therefore selective cloning into a single LTR is impossible. In order to add the Thy-1 sequence to the LTR subclones of each of the 3' LTR's from pLSN and pLGCTSN were made. For both pLSN and pLGCTSN the vectors are cut with the enzymes *Sal*I and *Xba*I, for pLSN this releases a fragment that is 268 base pairs long in the case of pLGCTSN the fragment is 679 bases in length. Each of these are in turn cloned into the *Sal*I and *Xba*I sites of pKSbluescript to create the subclones pks+3'LSN and pks+3'LGCTSN. To add the Thy sequence the partial LTR subclones are cut at the *Nhe*I site and the THY sequence that

has been cut out of pthyNhe using the enzymes *Nhe*I and *Xba*I is cloned into it. This then produces a second set of subclones pks+3'LSN+Thy and pks+3'LGCTSN+Thy. The partial LTR's containing the Thy elements can be moved into pLSNcat and pLGCTSNcat by cutting the LTR's out of the subclones at the *Sal*I and *Xba*I sites and cutting the recipient vectors at the reciprocal sites. This cloning results in pLGCTSNThy-cat and pLSNThy-cat. The identity of pks+3'LSN+Thy was confirmed by partial sequencing of this clone. The other clones were confirmed by restriction digestion.

C. Results

The Thy containing constructs were transfected into H9 cells and after one month of passage after selection the expression of CAT was assessed (cells were maintained with G418). We did not observe any difference in the cells containing the Thy constructs as compared to those that did not (Figure 23). The results in Figure 23 are from a cat assay from two different transfected and selected cell populations. H9 cells were used as they were a relevant cell type for HIV related experiments. After stable transfection the cell lines were held in culture and passaged every three days for 30 days until the assay time point. The standard CAT assay was used with 30 µl of cell lysate over a two hour reaction time. No positive increase in the stability of expression from the clones containing the Thy element were observed in this exercise. It was not determined whether the Thy element actually blocked methylation or not. Therefore it is not clear from this experiment if the Thy constructs failed because they did not block methylation or if blocking methylation is not adequate to stabilize expression levels in H9 cells.

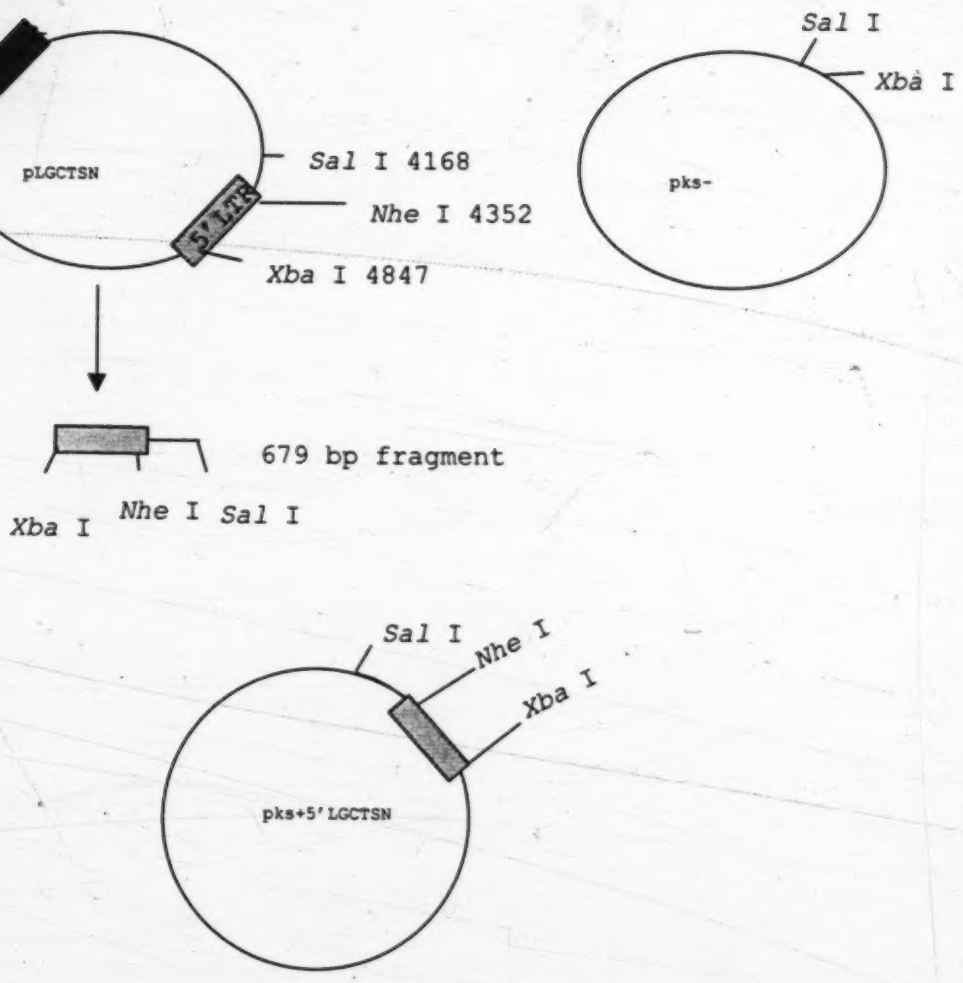


FIGURE 38. Construction strategy of pks+5'LGCTSN. The vector pLGCTSN is digested *Sal* I-*Xba* I to release a 679 bp fragment this is then cloned into the *Sal* I-*Xba* I sites of pks-.

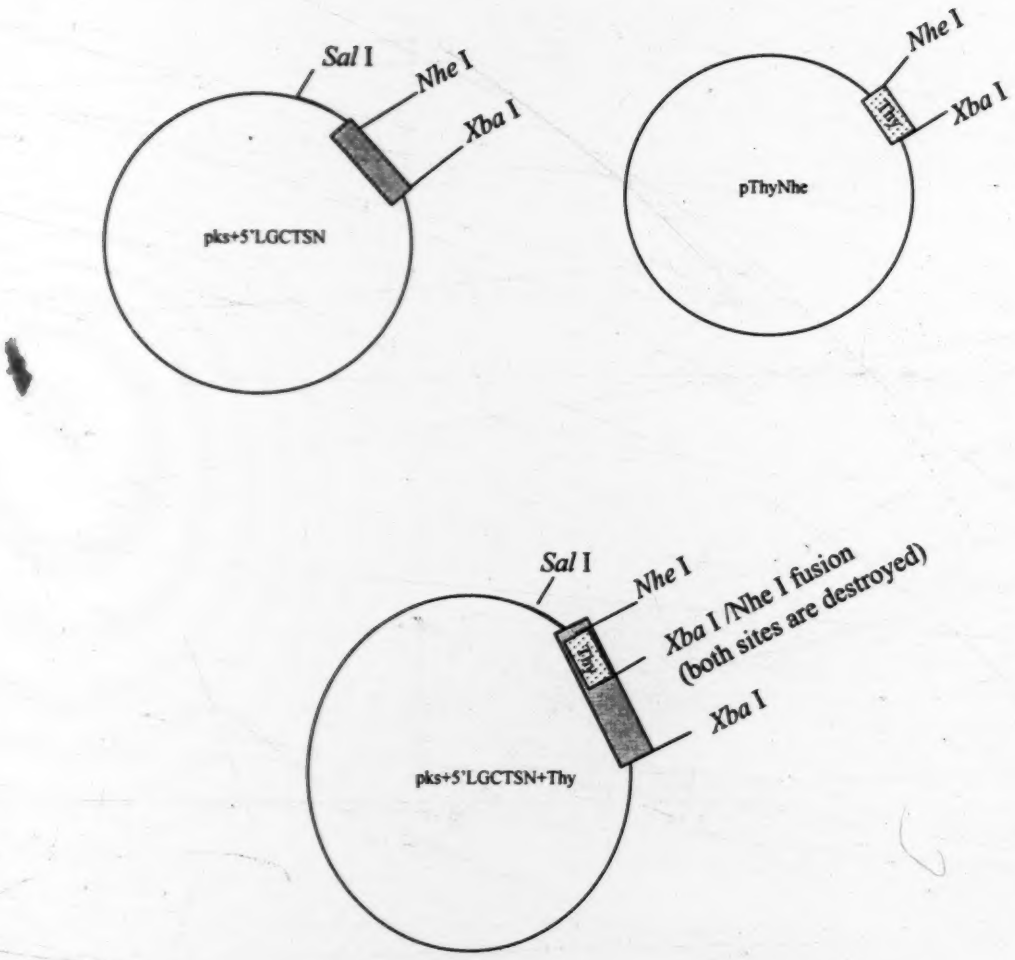


FIGURE 39. Construction strategy of pks+5'LGCTSN+Thy. The vector pks+5'LGCTSN is digested at the *Nhe* I site and a *Nhe* I-*Xba* I fragment from pThyNhe is added to the vector.

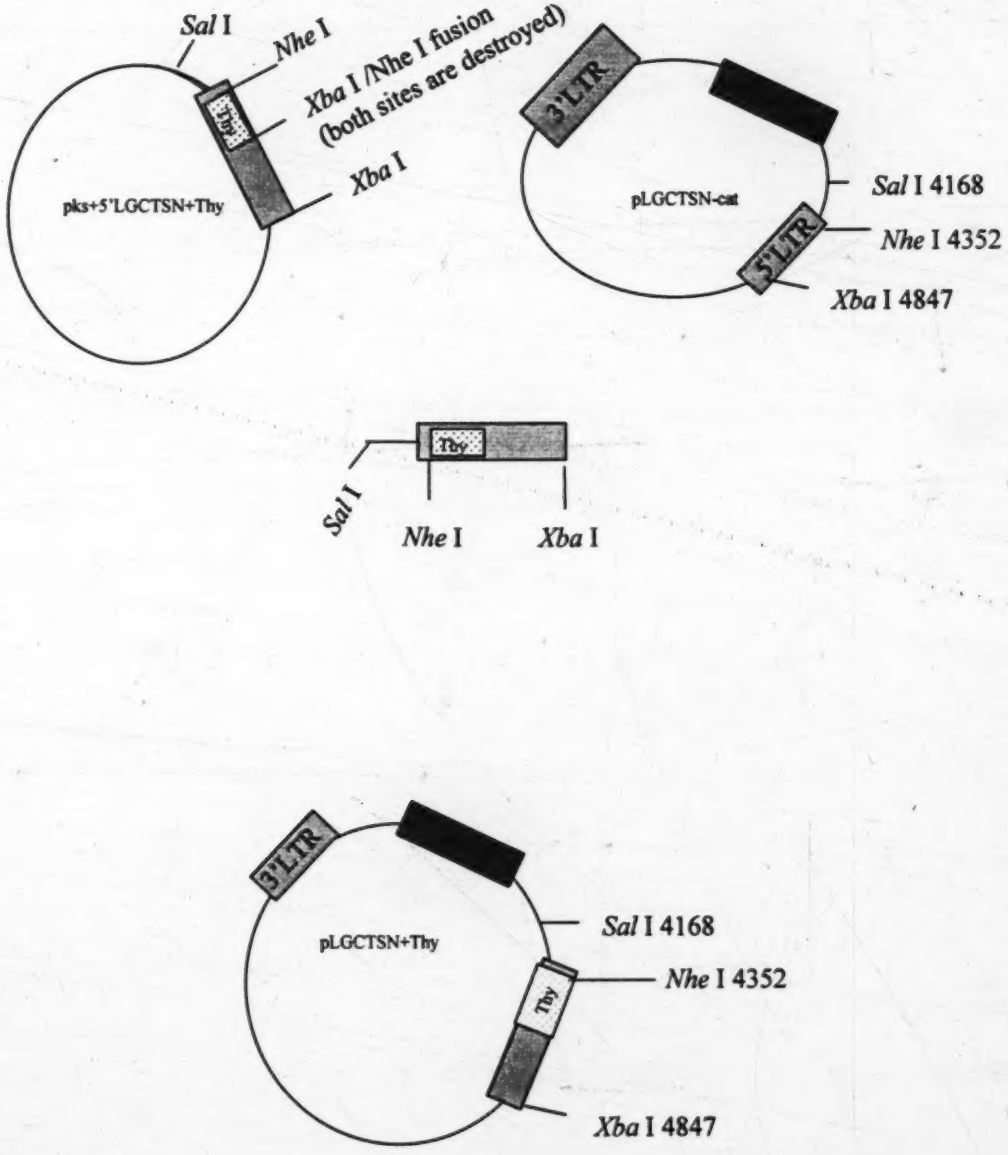
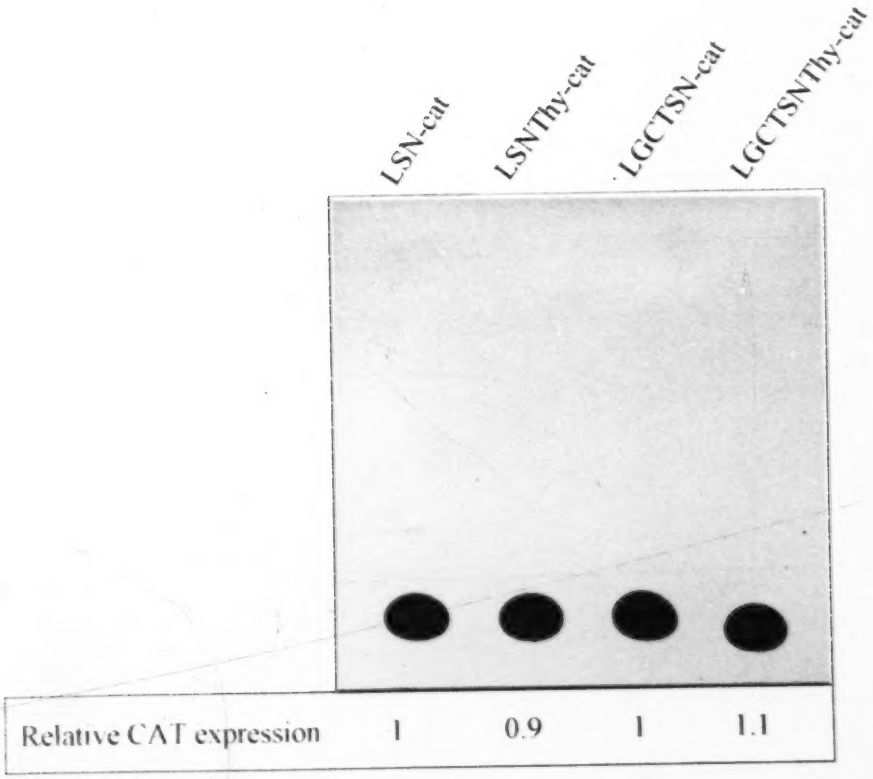


FIGURE 40. Construction strategy of pLGCTSN.Thy-cat. The vector pLGCTSN-cat is digested *Nhe* I-*Xba* I to release a 679 bp fragment. This fragment is replaced with the Thy containing LTR from pks+LGCTSN+Thy.

FIGURE 41. Analysis of the effect of the Thy-1 element on the long-term expression of retroviral vectors. Cells were transduced were LSN-cat, LSN.Thy-cat, LGCTSN-cat or LGCTSN.Thy-cat and selected in G418 for three weeks. The Cat was done on culture lysates 30 days after selection was determined to be complete. The cells were kept under selection for the entire time. The Cat assay was performed using cell lysates from pooled H9 transduced cells and was normalized for gross protein levels.

Promoter activities of Retroviral constructs
with or without the Thy-1 element in H9 cells



D. Discussion

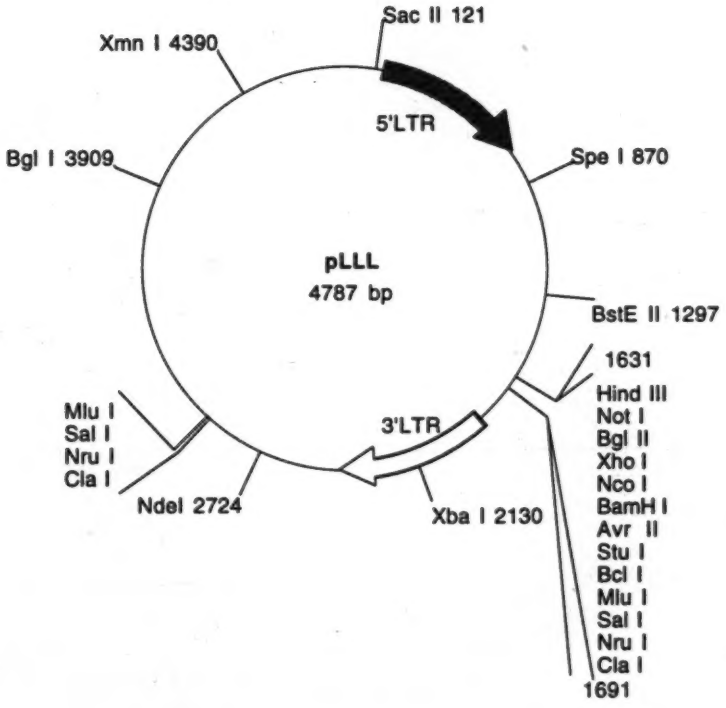
One explanation for a block in expression in genes transduced by retroviruses has been de novo methylation of the introduced promoter (59, 158). De novo methylation of cytosine residues has also been detected in embryonic cell lines (9). Methylation of cytosine residues results in a irreversible block to transcription initiation. In the vector system developed here, expression of introduced retroviral genes was only observed for 2-3 months. To address this, we attempted to produce a retroviral vector that would overcome the inherent inactivity of the MLV transcriptional unit in tissue culture cells. A region near the promoter of the Thy-1 gene protects itself as well as surrounding DNA sequences (338). This sequence has been reported to inhibit de novo methylation of adjacent heterologous sequences in a plasmid transfected into embryonic stem cells (338). A portion of this sequence (214 bases) that had been reported retain the function of the originating element was introduced into the 3' LTR of our gene therapy vectors (338).

The inclusion of the Thy-1 fragment did not increase the long term stability of transcription from the integrated LGCTSN. This element was included to inhibit methylation which has been shown to limit transcription from MLV based vectors. As this work did not produce a positive result it was not followed any further.

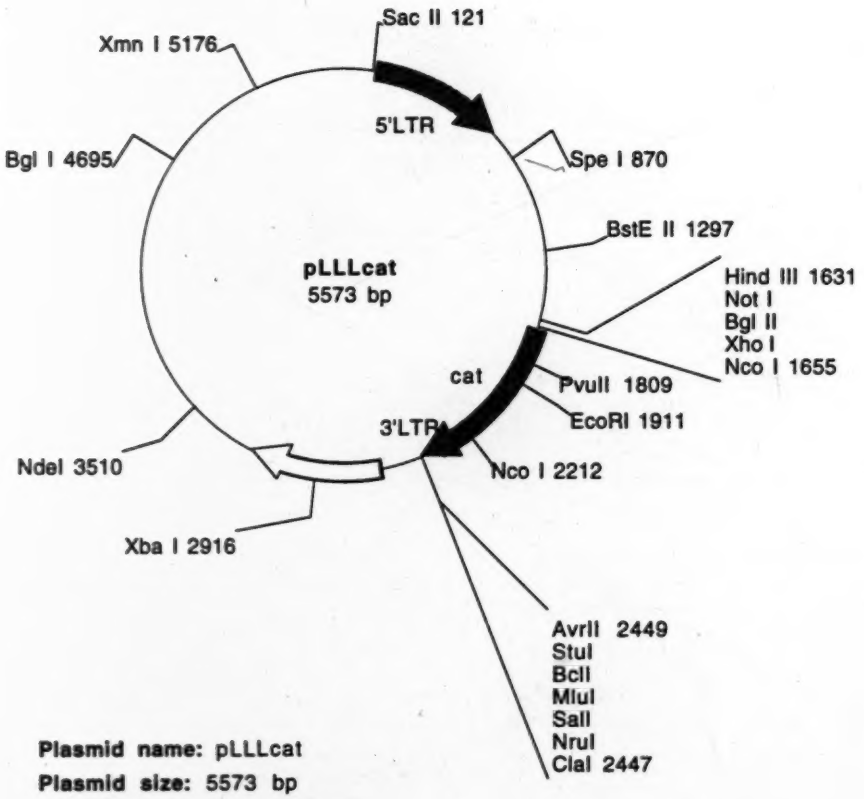
To determine why this addition did not yield improved performance one could investigate the initial premise that Thy-1 inhibited methylation. This could be done by analysing restriction patterns in Southern blots of the integrated provirus. For example, the retroviral LTR contains sites for the enzyme *Hpa*II. *Hpa*II is a methylation-sensitive enzyme and can be compared to a digest using the methylation insensitive isoschizomer *Msp* I.

APPENDIX B

MAPS OF CLONES



Plasmid name: pLLL
Plasmid size: 4787 bp
Constructed by: Lung-Ji Chang
Construction date: 1993
Comments/References: Derived from pLNL6



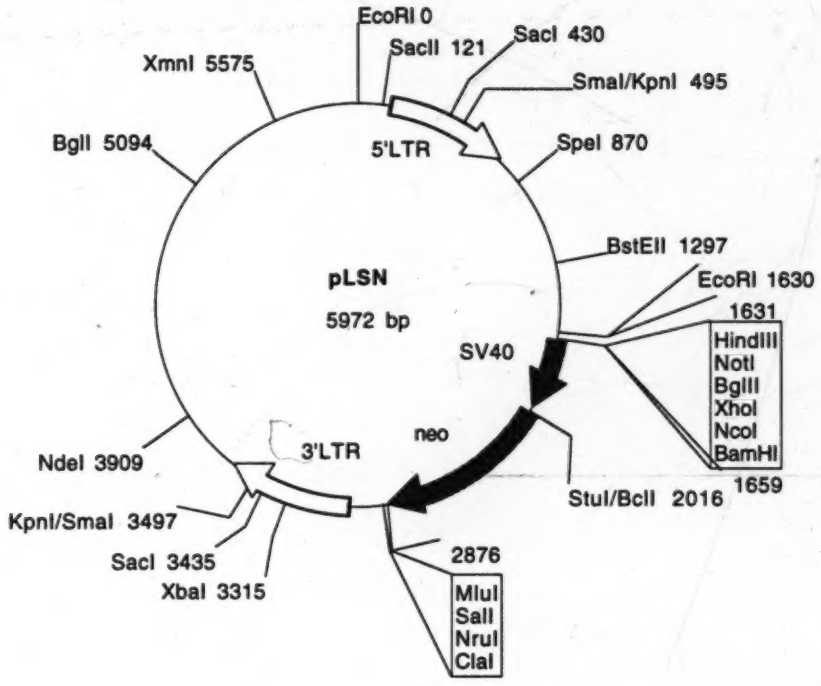
Plasmid name: pLLLcat

Plasmid size: 5573 bp

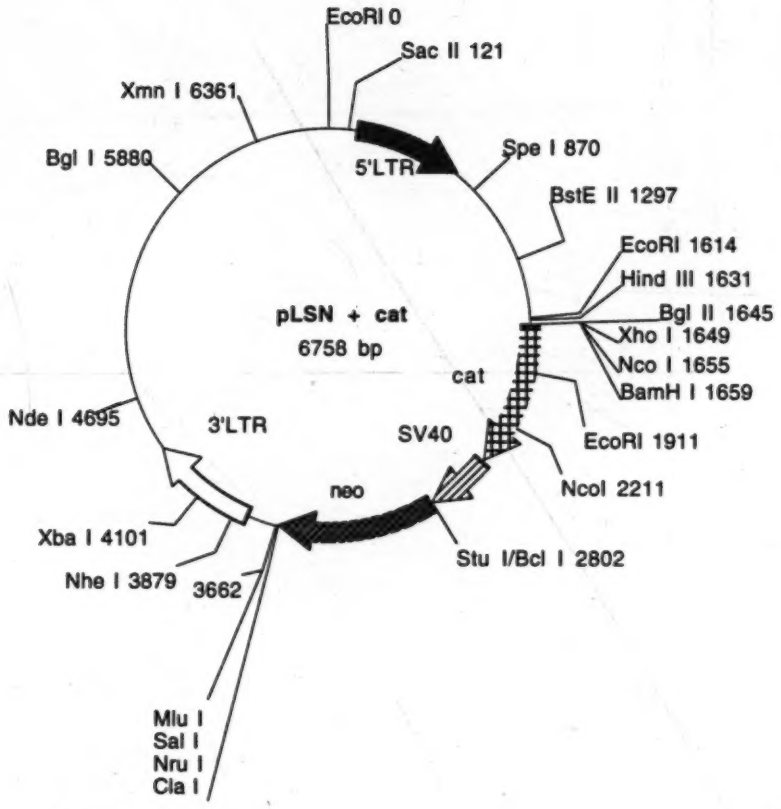
Constructed by: D. Robinson

Construction date: 1993

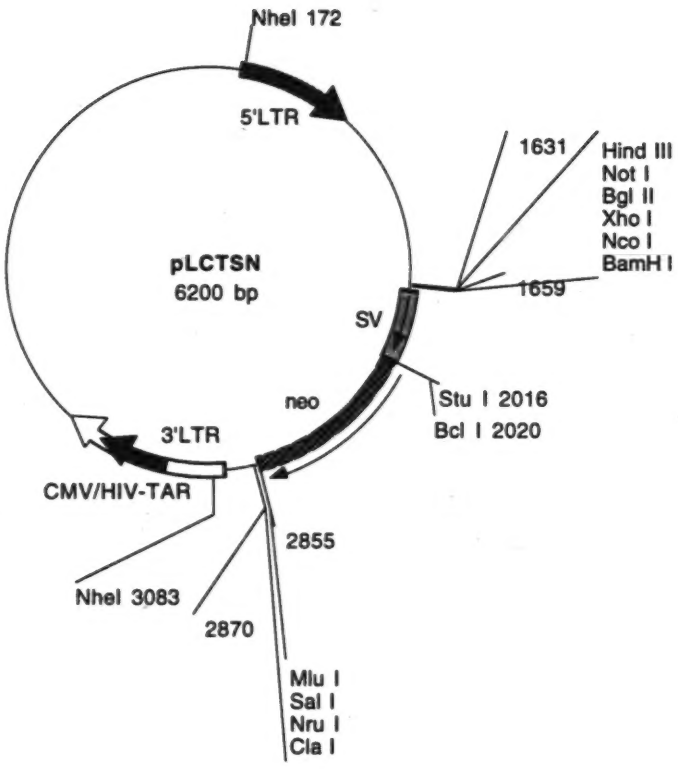
Comments/References: Derived from pLLL the vector is cut at BamHI and and treated with calf alkaline phosphatase. The Cat gene is cut out of pCAT at Sau3A1 and BglII and cloned into the BamHI site.



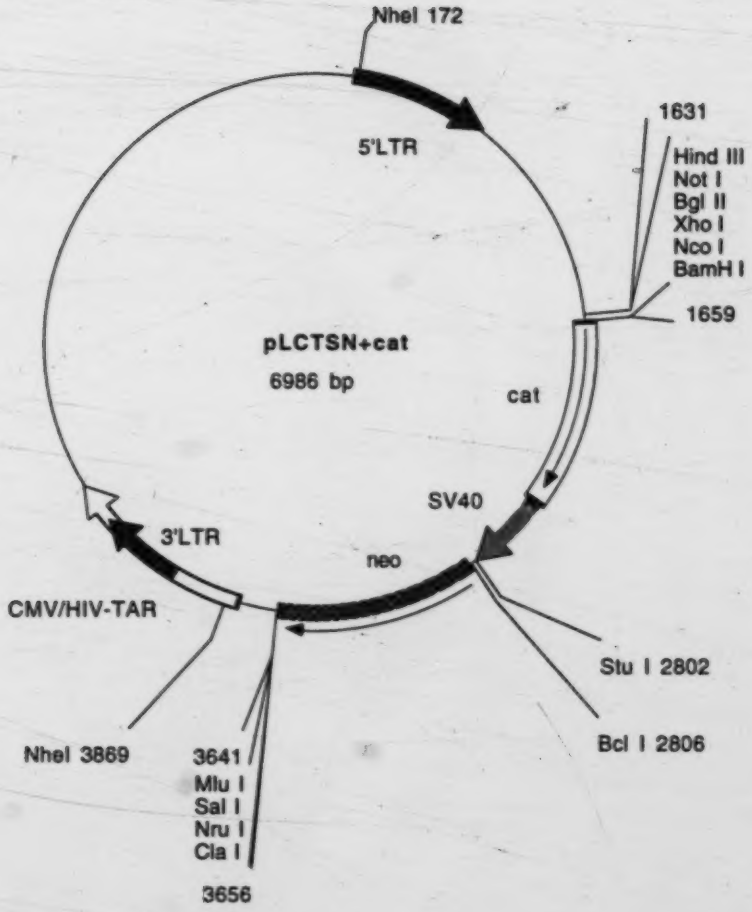
Plasmid name: pLSN
Plasmid size: 5972 bp
Constructed by: Lung-Ji Chang
Construction date: 1993
Comments/References: pLSN, derived from pLLLSV40



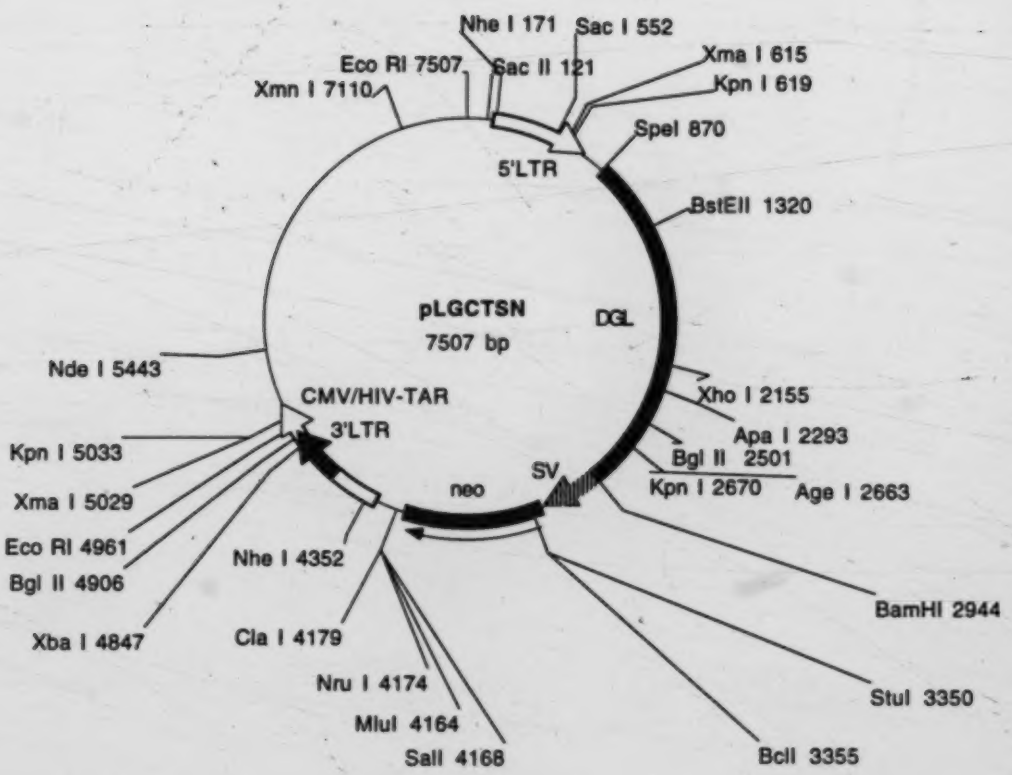
Plasmid name: pLSN + cat
Plasmid size: 6758 bp
Constructed by: D. Robinson
Construction date: 1993
Comments/References: The vector LSN is cut at BamHI and a 786 base pair piece of DNA contianing the Cat gene from pCAT cut BglII Sau3AI is cloned into the calf alkaline phosphatase treated site



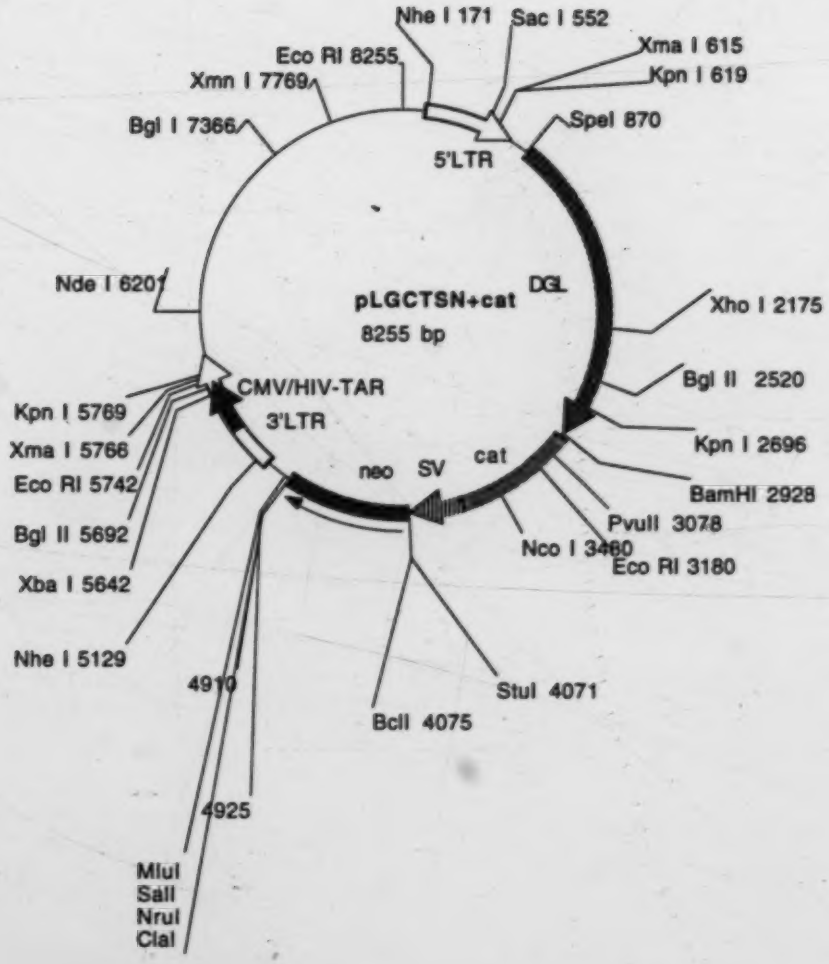
Plasmid name: pLCTSN
Plasmid size: 6200 bp
Constructed by: Lung-Ji Chang
Construction date: 1993
Comments/References: Derived from pLLL



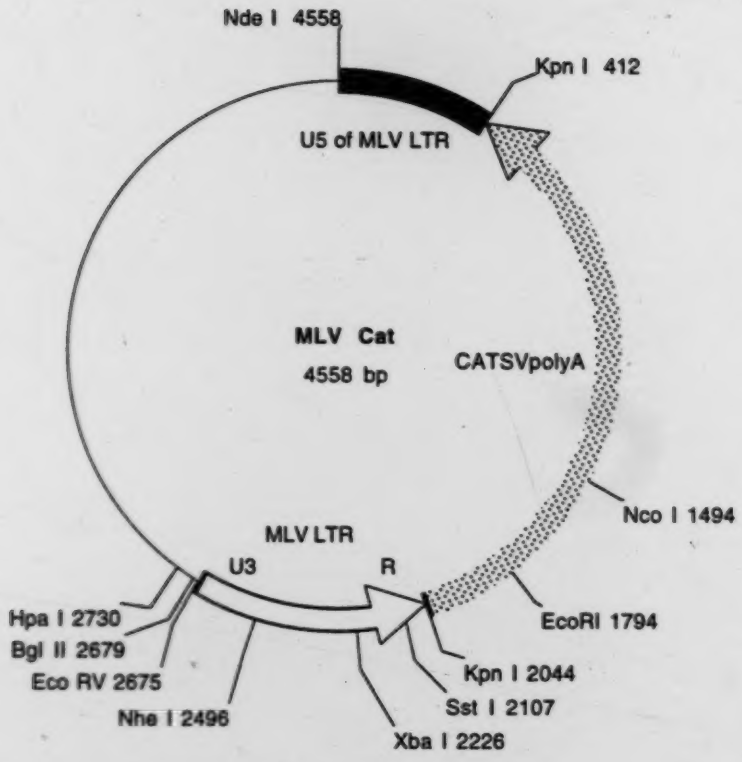
Plasmid name: pLCTSN+cat
Plasmid size: 6986 bp
Constructed by: D. Robinson
Construction date: 1993
Comments/References: The cat gene is bounded by Bam HI and Sau 3A1 sticky ends is inserted into the Bam H1 site of pLCTSN



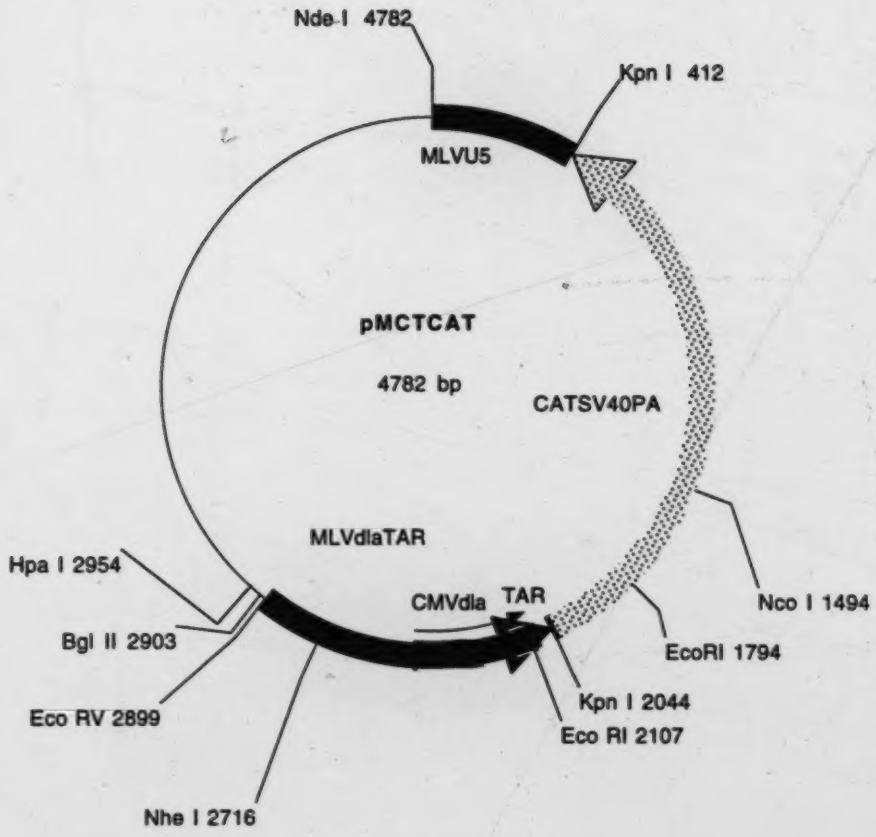
Plasmid name: pLGCTSN
Plasmid size: 7507 bp
Constructed by: Lung-Ji Chang
Construction date: 1993
Comments/References: Derived from pLLL



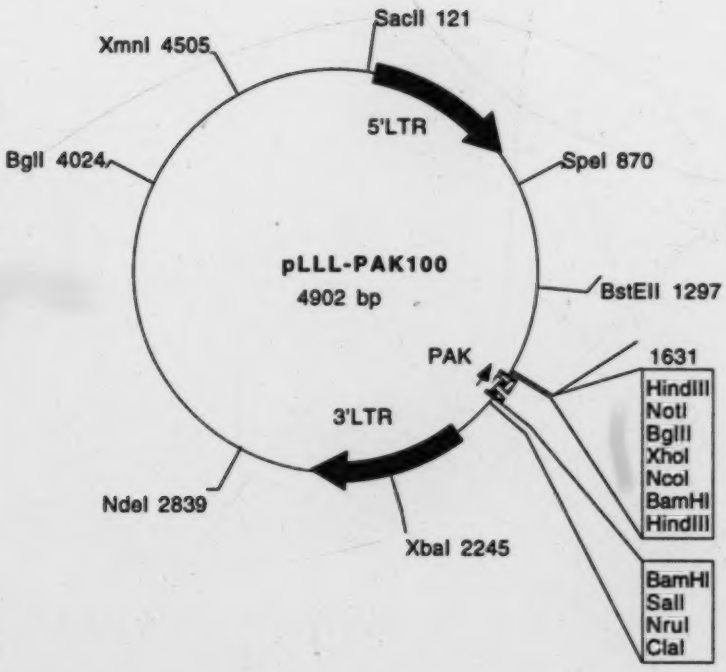
Plasmid name: pLGCTSN+cat
Plasmid size: 8255 bp
Constructed by: D. Robinson
Construction date: 1993
Comments/References: LGCTSN + cat



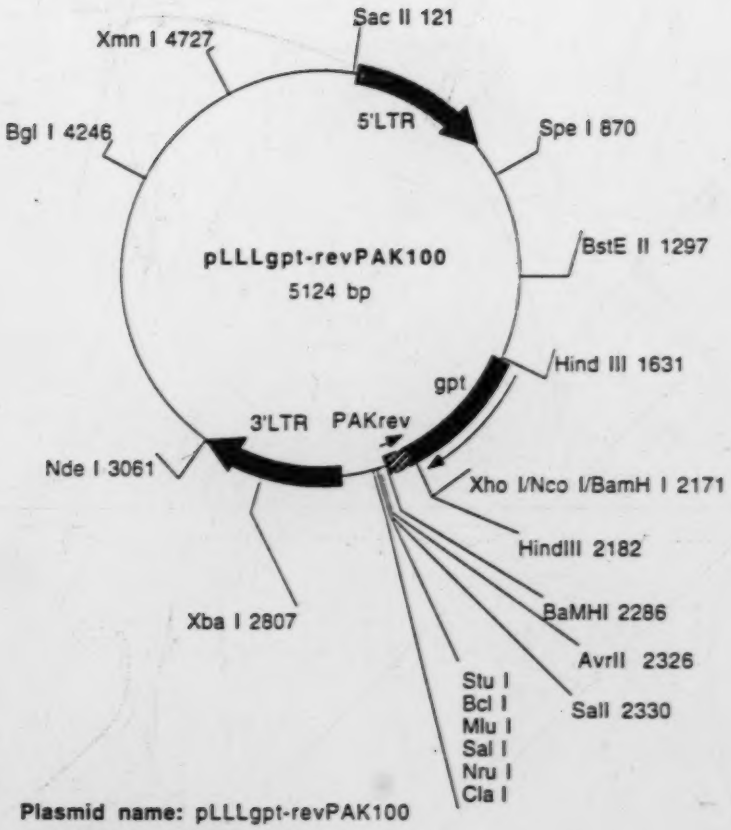
Plasmid name: MLV Cat
Plasmid size: 4558 bp
Constructed by: D. Robinson
Construction date: 1994
Comments/References: Derived from Sp72 NheI+KpnI=452+1632 EcoRI+KpnI=250+1382



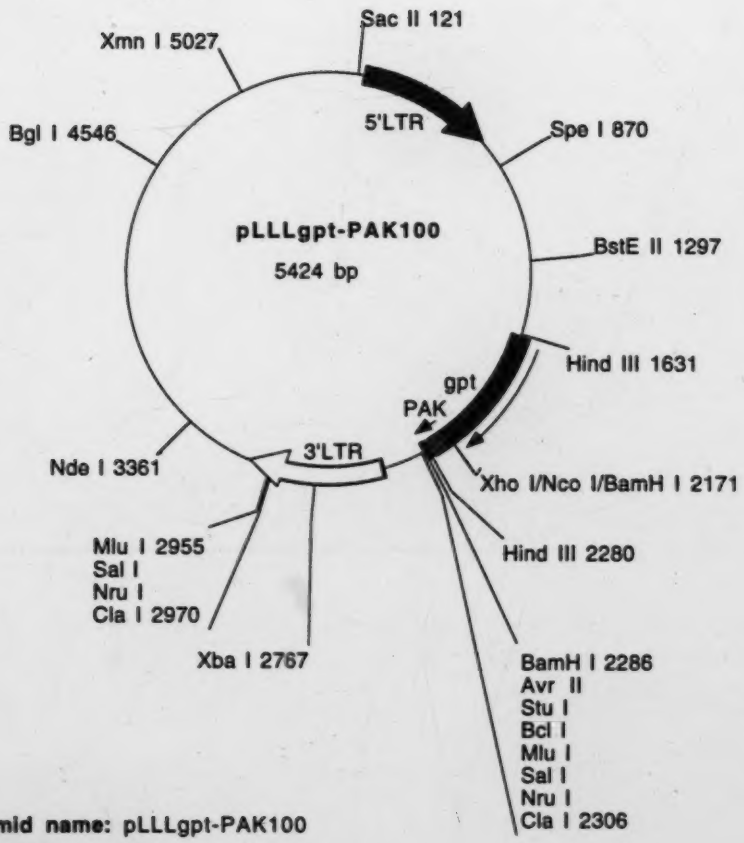
Plasmid name: pMCTCAT
Plasmid size: 4782 bp
Constructed by: Dr. L. Chang
Construction date: 1993
Comments/References: Derived from SP72 Nhe I + KpnI=672
Kpn I + RI=250+1382+63



Plasmid name: pLLL-PAK100
Plasmid size: 4902 bp
Constructed by: Deborah Robinson
Construction date: 1994
Comments/References: pLLL-PAK, derived from pLLL



Plasmid name: pLLLgpt-revPAK100
Plasmid size: 5124 bp
Constructed by: Deborah Robinson
Construction date: 1994
Comments/References: pLLLgptPAK100 is derived from pLLLgpt and pLLLPAK100 the PAK100 sequence is cut out of the LLLPAK100 vector using the flanking Bam HI sites and cloned into the Bam HI site of pLLLgpt.



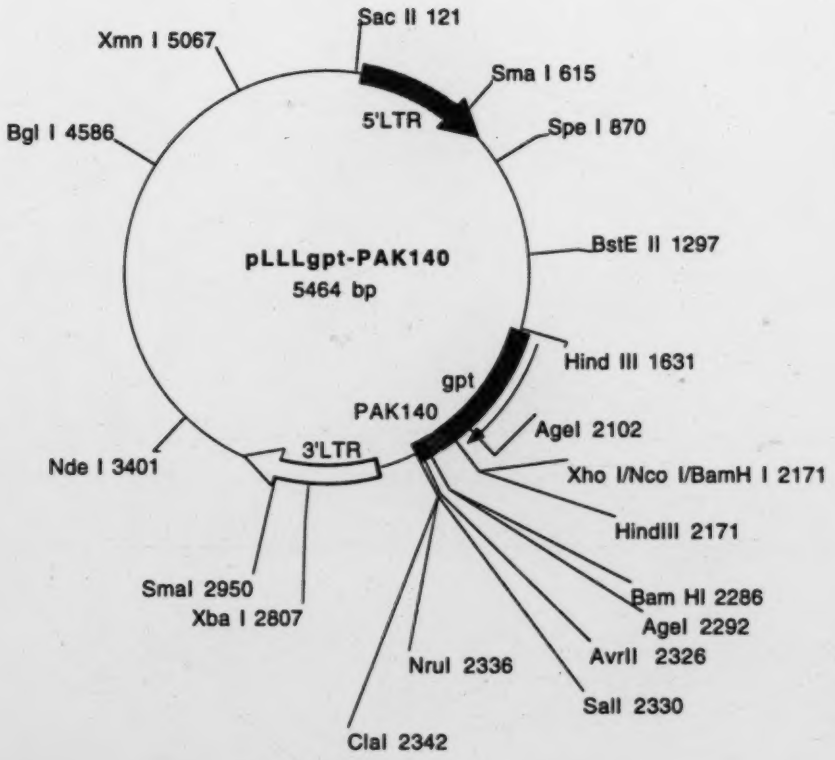
Plasmid name: pLLLgpt-PAK100

Plasmid size: 5424 bp

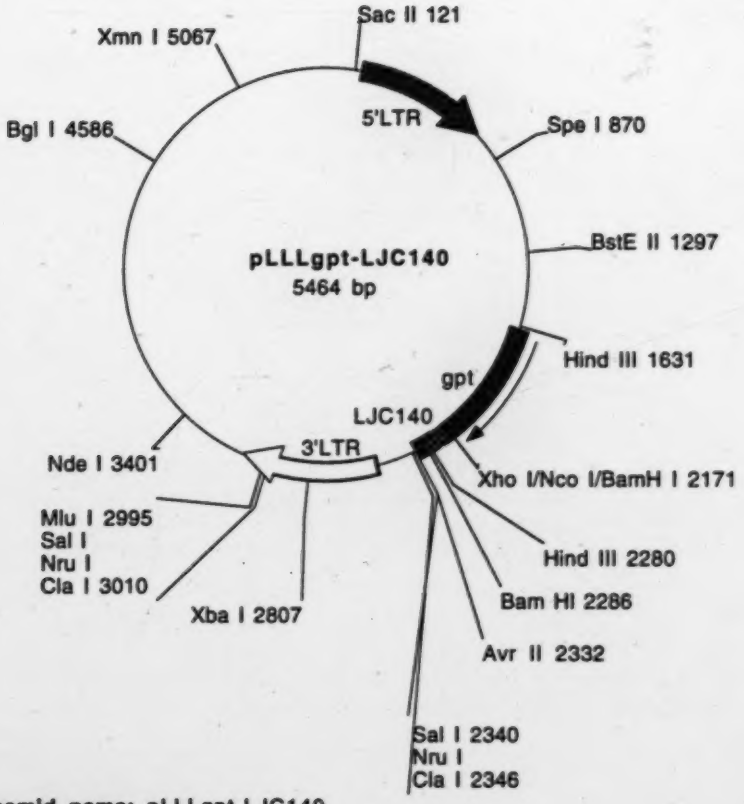
Constructed by: Deborah Robinson/Lung-Ji Chang

Construction date: 1994

Comments/References: pLLLgpt-PAK, derived from pLLLgpt and pLLL-PAK100 by digesting pLLL-PAK100 with Bam HI to release the PAK100 fragment and cloning into the Bam HI site of pLLLgpt.



Plasmid name: pLLLgpt-PAK140
Plasmid size: 5464 bp
Constructed by: Deborah Robinson
Construction date: 1994
Comments/References: pLLLgptrevPAK100+40, derived from pLLLgpt revPAK an oligo with forty bases of HIV gag are cloned into the AvrII and SalI site of vector. vector AvrII site is not retained but the oligo introduces a new one



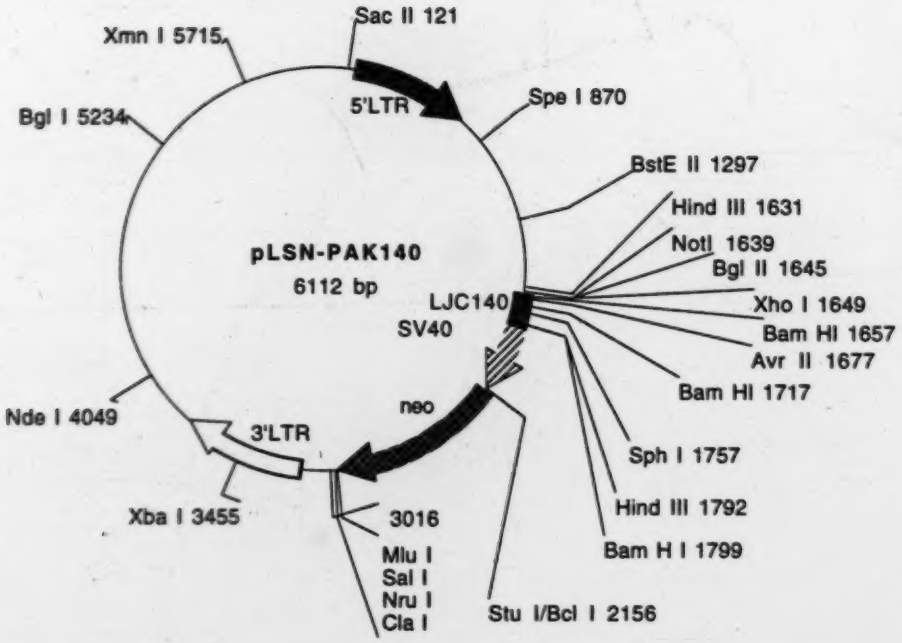
Plasmid name: pLLLgpt-LJC140

Plasmid size: 5464 bp

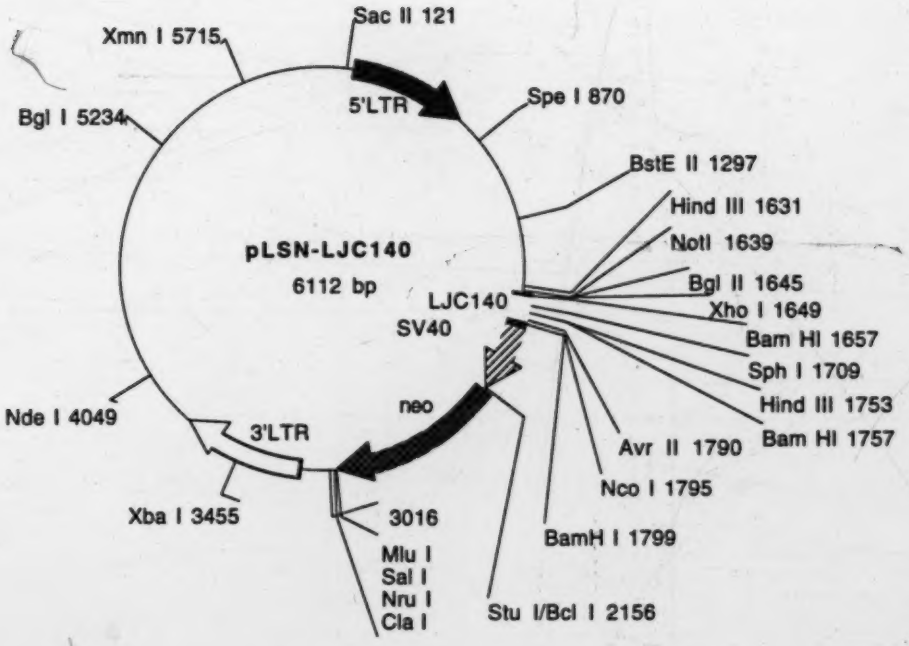
Constructed by: Lung-Ji Chang

Construction date: 1994

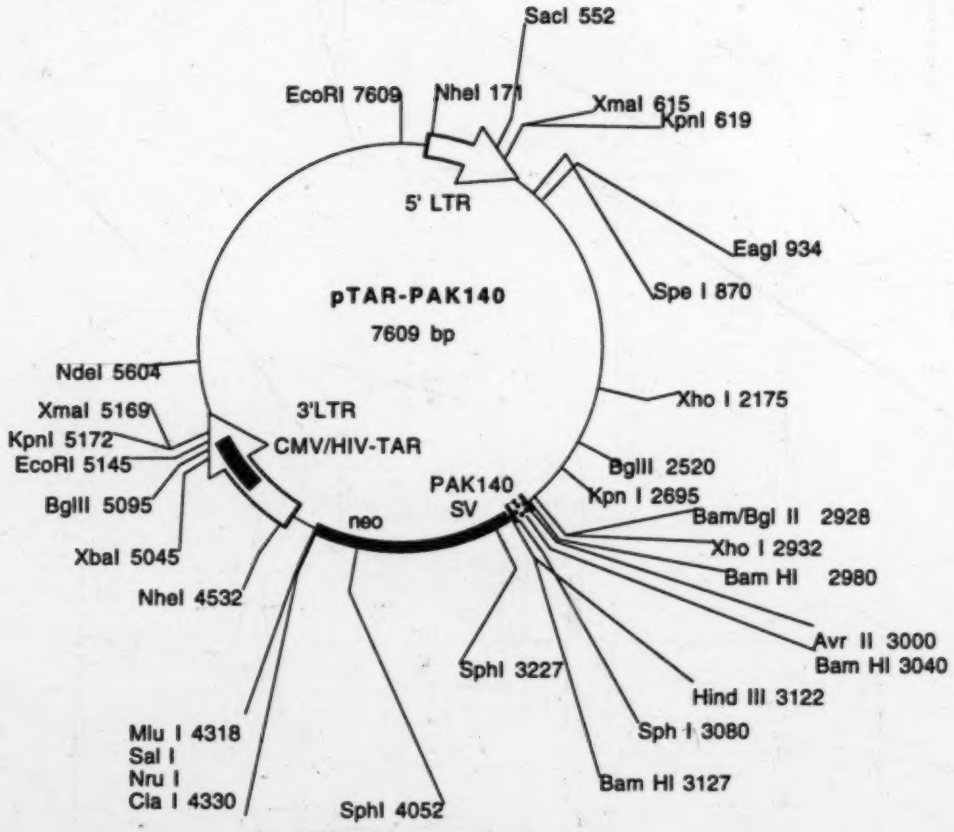
Comments/References: pLLLgptPAK140, derived from pLLLgpt and pLLL-PAK



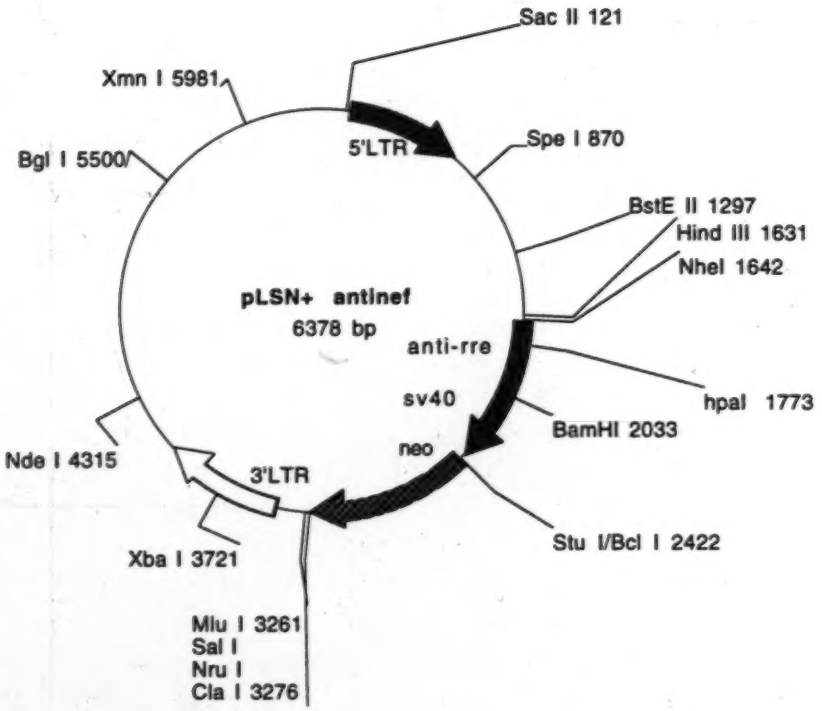
Plasmid name: pLSN-PAK140
Plasmid size: 6112 bp
Constructed by: D. Robinson
Construction date: 1993
Comments/References: pLSN- PAK140 derived from pLSN and pLLLgpt-PAK140



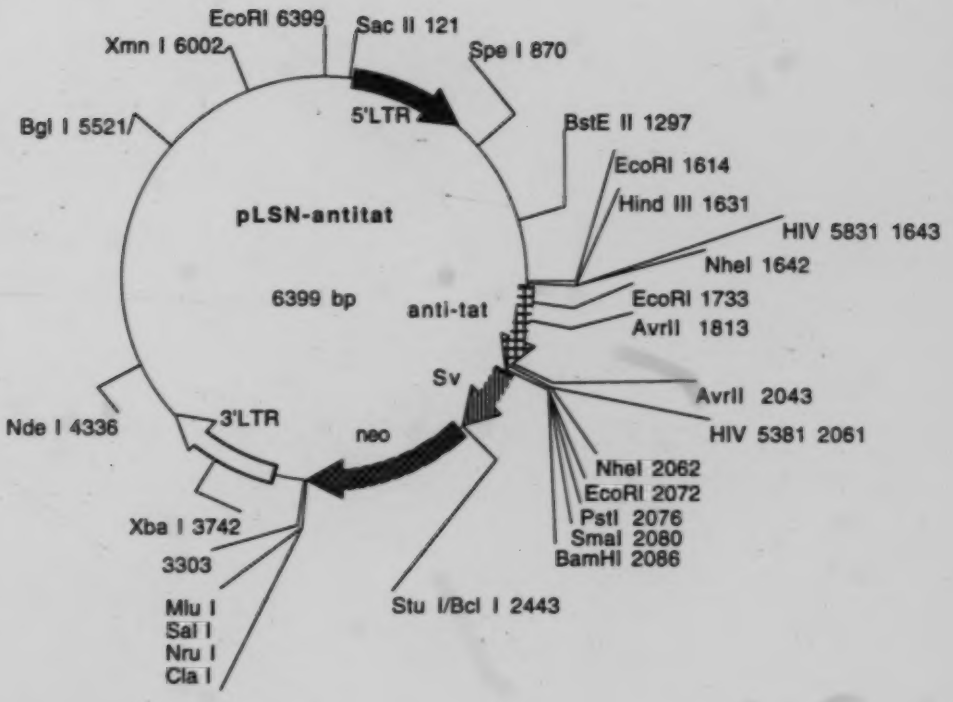
Plasmid name: pLSN-LJC140
Plasmid size: 6112 bp
Constructed by: Lung-Ji Chang
Construction date: 1993
Comments/References: pLSN+LJC140, is derived from pLSN and pLLLgpt-LJC140. Where the LJC140 fragment is digested Xho I to Sal I and cloned into the Xho I site of pLSN



Plasmid name: pTAR-PAK140
Plasmid size: 7609 bp
Constructed by: D Robinson
Construction date: 1995
Comments/References: This clone is derived from pLSNPAK-140, which is cut BglII to SalI to produce a fragment that is 1394 bases long. This fragment contains the Sv neo portion of the vector as well asPAK140. It is inserted into a BamHI to SalI cut on the vector pLGCTSN.



Plasmid name: pLSN+ antinef
Plasmid size: 6378 bp
Constructed by: robinson
Construction date: 1995
Comments/References: pLSNcut HindIII/BAMHI KS anti nef cut HindIII/BamHI



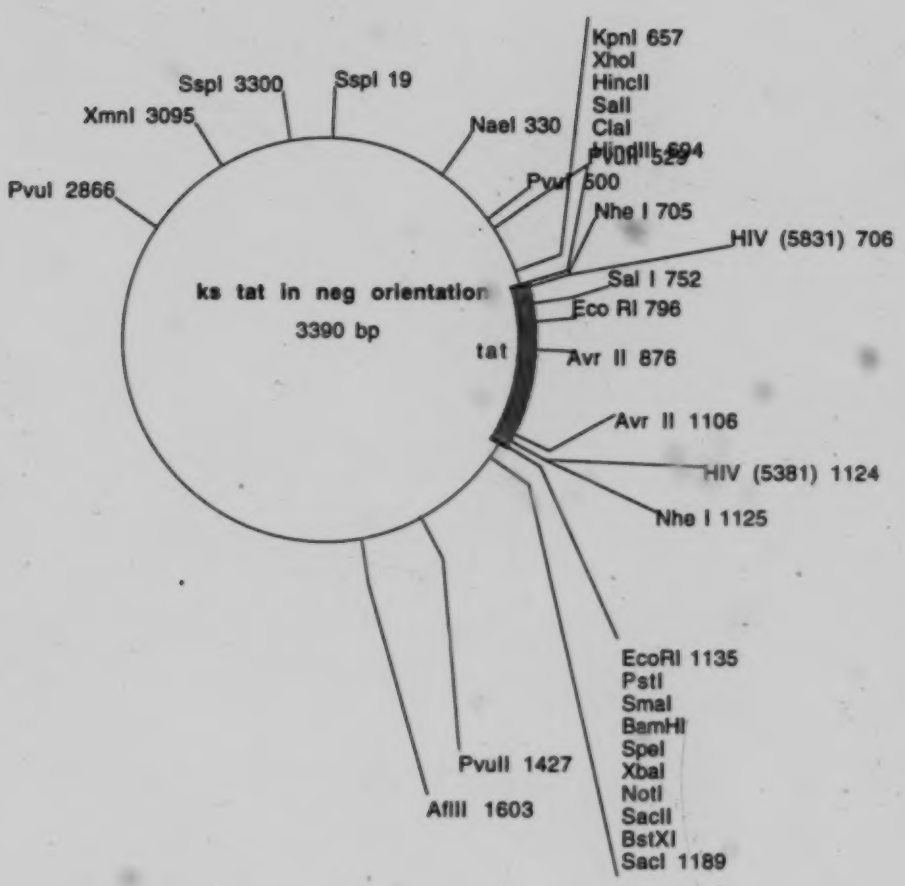
Plasmid name: pLSN-antitat

Plasmid size: 6399 bp

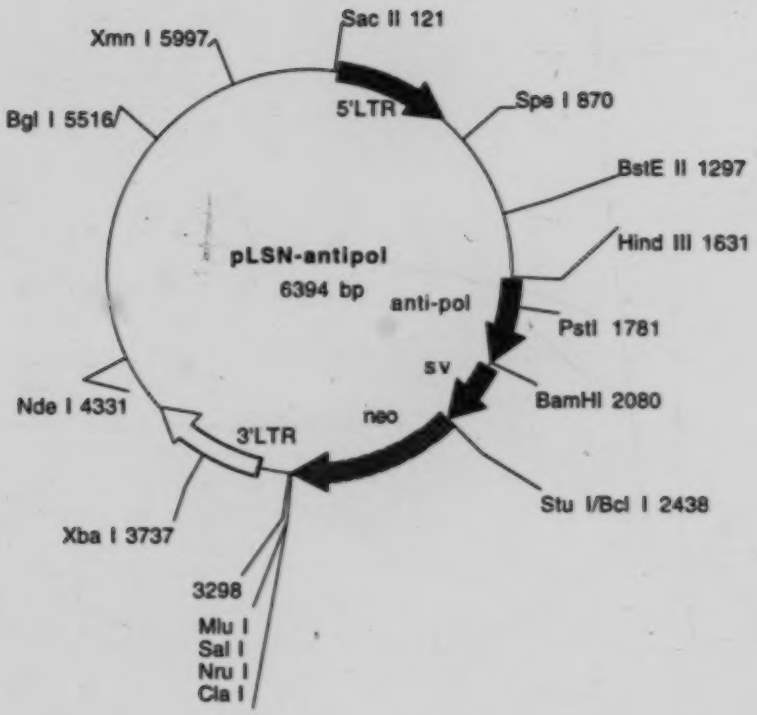
Constructed by: D. Robinson

Construction date: 1995

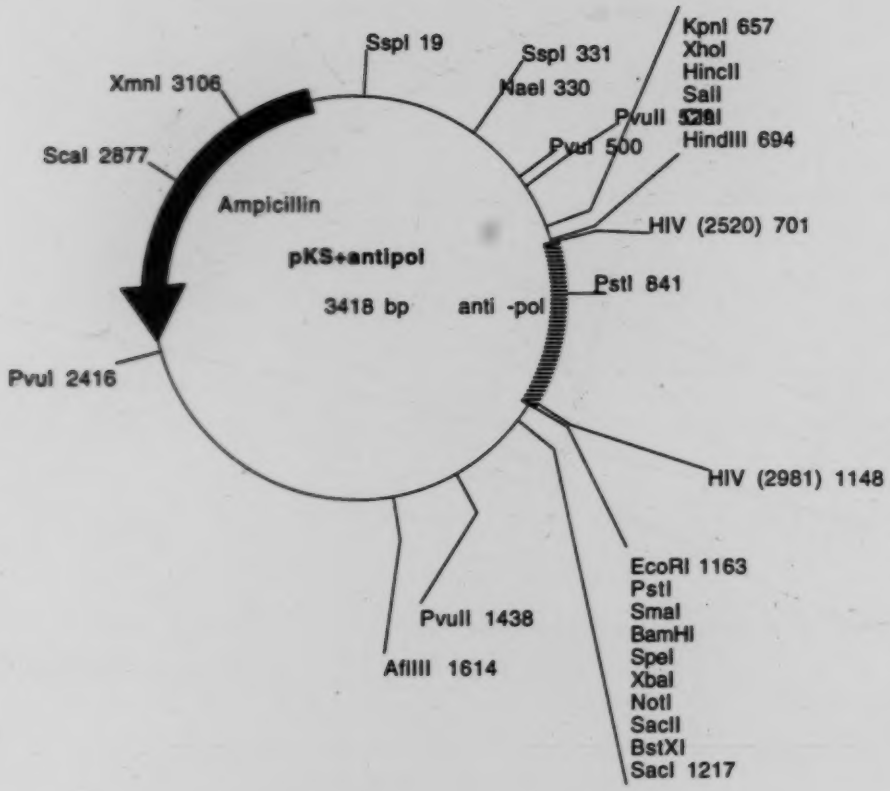
Comments/References: pLSNantitat is made by cutting the vector LSN at the BamHI and HindIII sites and cloning into these sites a BamHI and HindIII fragment from pkstat a subclone which contains the PCR fragment of HIV(5831-5381).



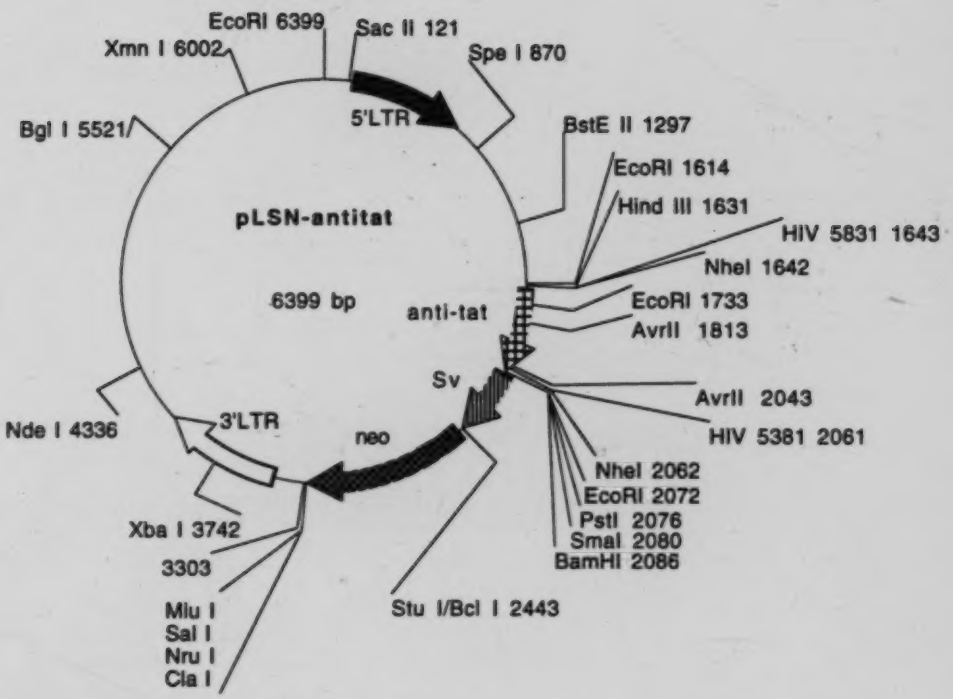
Plasmid name: ks tat in neg orientation
Plasmid size: 3390 bp
Constructed by: D. Robinson
Construction date: July 1995
Comments/References: The insert shown in this plasmid is a PCR product of the HIV-1 (5381-5831) genome. To produce pks+tat the insert was blunt cloned into the EcoRV site of the originating vector pks(bluescript).



Plasmid name: pLSN-antipol
Plasmid size: 6394 bp
Constructed by: D.Robinson
Construction date: 1995
Comments/References: The vector pLSN is cut HindIII to BamHI. The insert containing anti-pol is removed from pks+ anti-pol at the HindIII and BamHI sites and is cloned into the vector at the complimentary cut sites.



Plasmid name: pKS+antipol
Plasmid size: 3418 bp
Constructed by: D Robinson
Construction date: july 1995
Comments/References: This clone was made by cutting pNL4-3(HIV-1); withHInc II at HIV map position 2520 and EcoRV at 2981 and cloning this fragment into the EcoRV site of bluescript. Orientation is correct as shown.



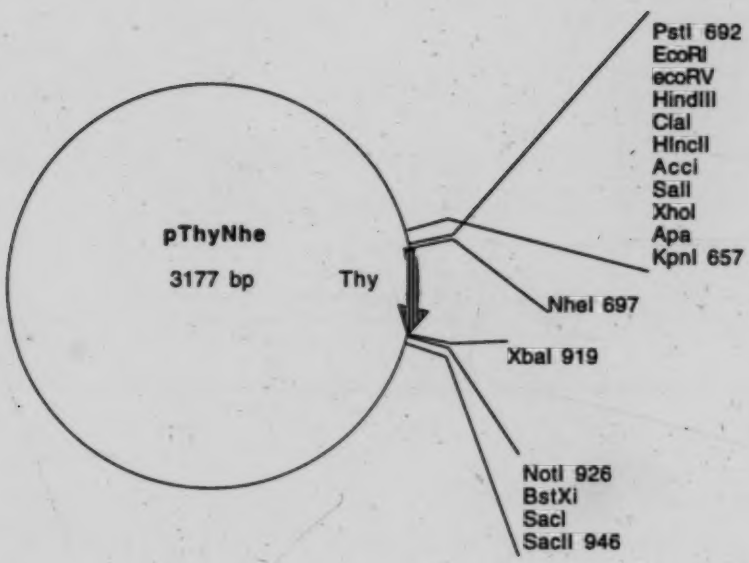
Plasmid name: pLSN-antitat

Plasmid size: 6399 bp

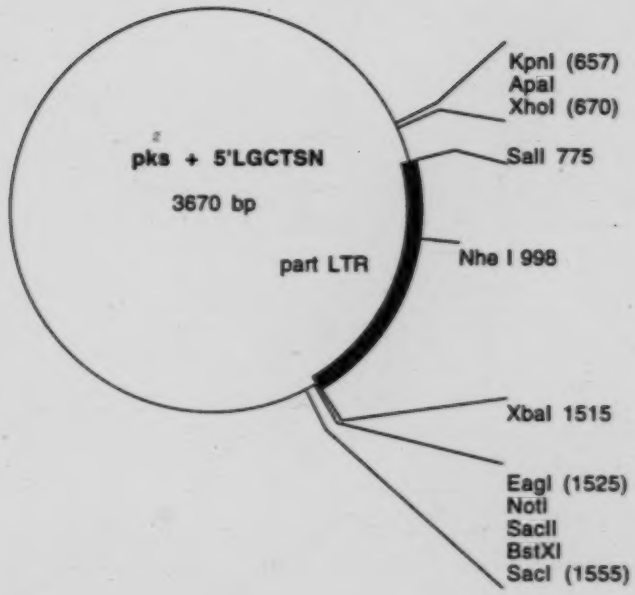
Constructed by: D. Robinson

Construction date: 1995

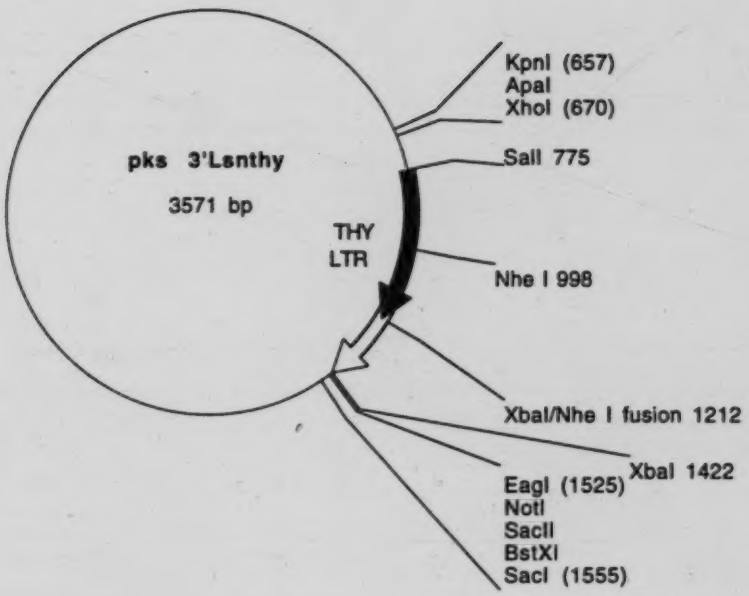
Comments/References: pLSNantitat is made by cutting the vector LSN at the BamHI and HindIII sites and cloning into these sites a BamHI and HindIII fragment from pkstat a subclone which contains the PCR fragment of HIV(5831-5381).



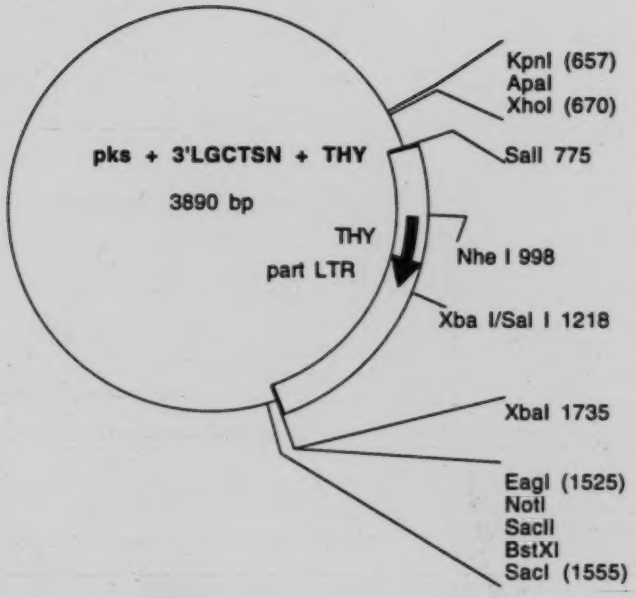
Plasmid name: pThyNhe
Plasmid size: 3177 bp
Constructed by: D. Robinson
Construction date: 1994
Comments/References: pksTHYNhe is made from pksTHY by cutting the vector at the Sma I site and ligating in a linker fragment containing the Nhe I site.



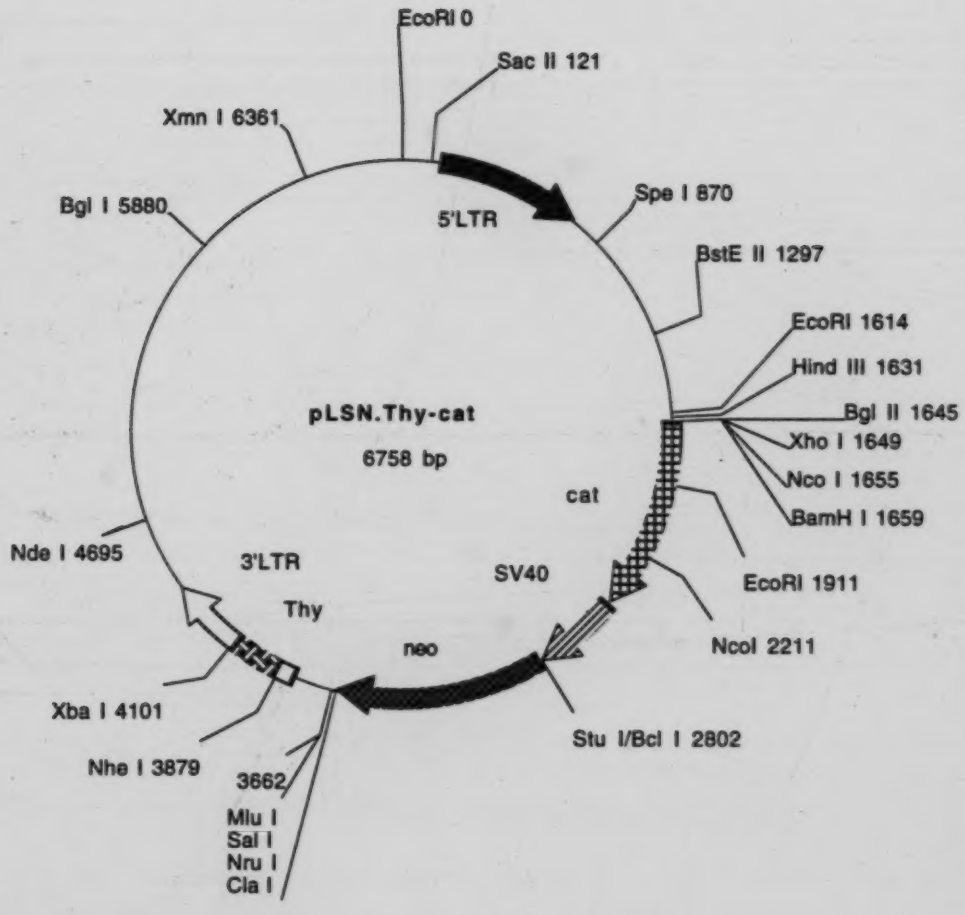
Plasmid name: pks + 5'LGCTSN
Plasmid size: 3670 bp
Constructed by: D. Robinson
Construction date: 1994
Comments/References: A portion of the 3'LTR from pLGCTSN is removed by cutting on the Sal I and Xbal site. The LTR containing fragment is then cloned into the pks(bluescript) cut at the reciprocal sites.



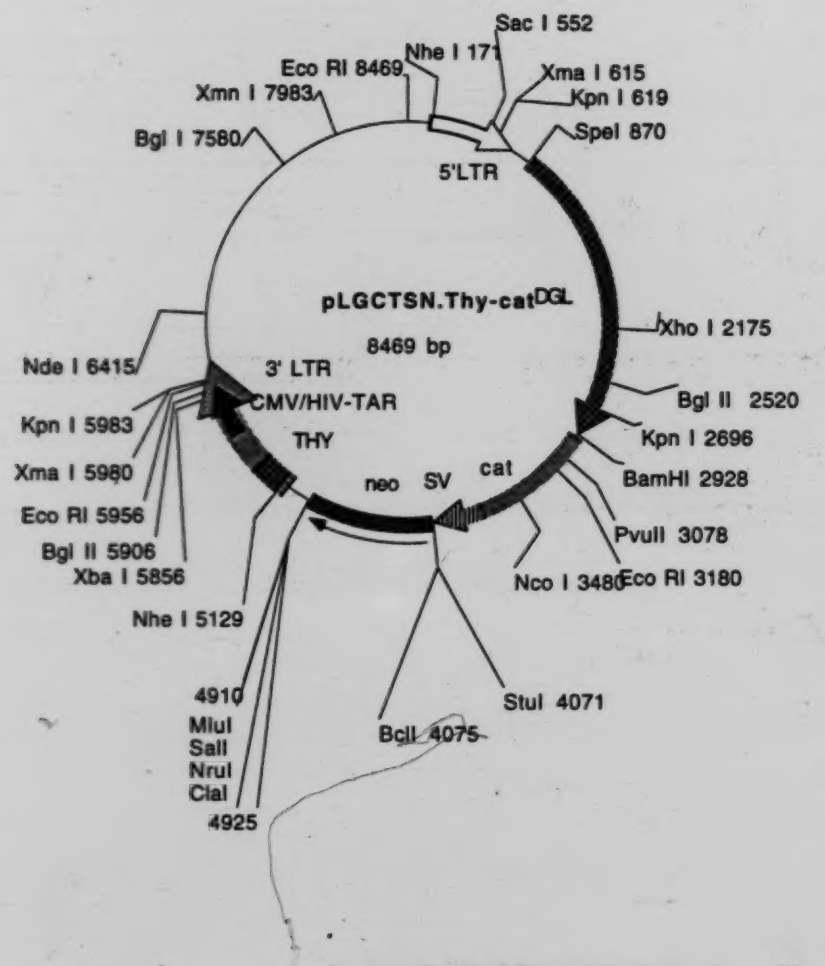
Plasmid name: pks 3'Lsnthy
Plasmid size: 3571 bp
Constructed by: D. Robinson
Construction date: 1994
Comments/References: The subcloned LTR contained inpKS3'LSN is Cut at the NheI site and the Thy fragment from pThyNhe is cut, Nhe I to Xba I and cloned into the opened vector.



Plasmid name: pks + 3'LGCTSN + THY
Plasmid size: 3890 bp
Constructed by: D. Robinson
Construction date: 1994
Comments/References: The Thy-1 demethylation sequence is added to subclone pks+3'LGCTSN by cutting the subclone at the NheI site and cloning in a NheI/Xba I fragment derived from the pTHyNhe clone



Plasmid name: pLSN.Thy-cat
Plasmid size: 6758 bp
Constructed by: D. Robinson
Construction date: 1995
Comments/References: LSNcat is cut at the Sal I and Xba I sites and this piece is replaced with the Sal I and XbaI fragment from the pksLSNTHY clone



Plasmid name: pLGCTSN.Thy-cat

Plasmid size: 8469 bp

Constructed by: D. Robinson

Construction date: 1995

Comments/References: LGCTSNCAT is cut at the Sal I and XbaI sites and this fragement is replaced with the Sal I and XbaI fragment of skLGCTSNTHY